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(54) Alpha-1,4-glucan lyase from a fungus infected algae, its purification, gene cloning and expression in microorganisms

(57) A method of preparing α -1,4-glucan lyase enzymes is described. The method comprises isolating the enzymes from a fungally infected algae. The amino acid sequences of the enzymes have been determined. The nucleic acid sequences coding for the enzymes have also been determined.

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(54) Title: ALPHA 1,4 GLUCAN LYASE FROM A FUNGUS INFECTED ALGAE, ITS PURIFICATION, GENE CLONING AND EXPRESSION IN MICROORGANISMS (57) Abstract <p>A method of preparing α-1,4-glucan lyase enzymes is described. The method comprises isolating the enzymes from a fungally infected algae. The amino acid sequences of the enzymes have been determined. The nucleic acid sequences coding for the enzymes have also been determined.</p>		

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ALPHA-1,4-GLUCAN LYASE FROM A FUNGUS INFECTED ALGAE, ITS PURIFICATION, GENE CLONING AND EXPRESSION IN MICROORGANISMS

The present invention relates to an enzyme, in particular α -1,4-glucan lyase ("GL").
The present invention also relates to a method of extracting the same. The present
invention also relates to nucleotide sequence(s) encoding for the same.

FR-A-2617502 and Baute et al in Phytochemistry [1988] vol. 27 No.11 pp3401-3403
report on the production of 1,5-D-anhydrofructose ("AF") in *Morchella vulgaris* by
an apparent enzymatic reaction. The yield of production of AF is quite low. Despite
a reference to a possible enzymatic reaction, neither of these two documents presents
any amino acid sequence data for any enzyme, let alone any nucleotide sequence
information. These documents say that AF can be a precursor for the preparation of
the antibiotic pyrone microthecin.

Yu et al in Biochimica et Biophysica Acta [1993] vol 1156 pp313-320 report on the
preparation of GL from red seaweed and its use to degrade α -1,4-glucan to produce
AF. The yield of production of AF is quite low. Despite a reference to the enzyme
GL this document does not present any amino acid sequence data for that enzyme let
alone any nucleotide sequence information coding for the same. This document also
suggests that the source of GL is just algal.

According to the present invention there is provided a method of preparing the
enzyme α -1,4-glucan lyase comprising isolating the enzyme from a fungally infected
algae.

Preferably the enzyme is isolated and/or further purified using a gel that is not
degraded by the enzyme.

Preferably the gel is based on dextrin, preferably beta-cyclodextrin, or derivatives
thereof, preferably a cyclodextrin, more preferably beta-cyclo-dextrin.

According to the present invention there is also provided a GL enzyme prepared by the method of the present invention.

Preferably the enzyme comprises the amino acid sequence SEQ. ID. No. 1. or SEQ. ID. No. 2, or any variant thereof.

The term "any variant thereof" means any substitution of, variation of, modification of, replacement of, deletion of or addition of at least one amino acid from or to the sequence providing the resultant enzyme has lyase activity.

According to the present invention there is also provided a nucleotide sequence coding for the enzyme α -1,4-glucan lyase, preferably wherein the sequence is not in its natural environment (i.e. does not form part of the natural genome of a cellular organism expressing the enzyme).

Preferably the nucleotide sequence is a DNA sequence.

Preferably the DNA sequence comprises a sequence that is the same as, or is complementary to, or has substantial homology with, or contains any suitable codon substitution(s) for any of those of, SEQ. ID. No. 3 or SEQ. ID. No. 4.

The expression "substantial homology" covers homology with respect to structure and/or nucleotide components and/or biological activity.

The expression "contains any suitable codon substitutions" covers any codon replacement or substitution with another codon coding for the same amino acid or any addition or removal thereof providing the resultant enzyme has lyase activity.

In other words, the present invention also covers a modified DNA sequence in which at least one nucleotide has been deleted, substituted or modified or in which at least one additional nucleotide has been inserted so as to encode a polypeptide having the activity of a glucan lyase, preferably an enzyme having an increased lyase activity.

According to the present invention there is also provided a method of preparing the enzyme α -1,4-glucan lyase comprising expressing the nucleotide sequence of the present invention.

5 According to the present invention there is also provided the use of beta-cyclodextrin to purify an enzyme, preferably GL.

According to the present invention there is also provided a nucleotide sequence wherein the DNA sequence comprises a sequence that is the same as, or is
10 complementary to, or has substantial homology with, or contains any suitable codon substitutions for any of those of, SEQ. ID. No. 3 or SEQ. ID. No. 4, preferably wherein the sequence is in isolated form.

A key aspect of the present invention is the recognition that GL is derived from a
15 fungally infected algae. This is the first time that the amino acid sequence of GL has been determined in addition to the determination of the nucleic acid sequences that code for GL. A key advantage of the present invention is therefore that GL can now be made in large quantities by for example recombinant DNA techniques and thus enable compounds such as the antibiotic microthecin to be made easily and in larger
20 amounts.

The enzyme should preferably be secreted to ease its purification. To do so the DNA encoding the mature enzyme is fused to a signal sequence, a promoter and a
25 terminator from the chosen host.

For expression in *Aspergillus niger* the *gpdA* (from the Glyceraldehyde-3-phosphate dehydrogenase gene of *Aspergillus nidulans*) promoter and signal sequence is fused to the 5' end of the DNA encoding the mature lyase - such as SEQ I.D. No. 3 or
30 SEQ. I.D. No.4. The terminator sequence from the *A. niger trpC* gene is placed 3' to the gene (Punt, P.J. et al (1991): J. Biotech. 17, 19-34). This construction is inserted into a vector containing a replication origin and selection origin for *E. coli* and a selection marker for *A. niger*. Examples of selection markers for *A. niger* are

the amdS gene, the argB gene, the pyrG gene, the hygB gene, the BmlR gene which all have been used for selection of transformants. This plasmid can be transformed into *A. niger* and the mature lyase can be recovered from the culture medium of the transformants.

5

The construction can be transformed into a protease deficient strain to reduce the proteolytic degradation of the lyase in the culture medium (Archer D.B. et al (1992): Biotechnol. Lett. 14, 357-362).

10

Other advantages will become apparent in the light of the following description.

15

The present invention therefore relates to the isolation of the enzyme α -1,4-glucan lyase from a fungus infected algae - preferably a fungus infected red algae such as the type that can be collected in China - such as *Gracilariopsis lemaneiformis*. An example of a fungally infected algae has been deposited in accordance with the Budapest Treaty (see below).

20

By using *in situ* hybridisation technique it was established that the enzyme GL was detected in the fungally infected red algae *Gracilariopsis lemaneiformis*. Further evidence that supports this observation was provided by the results of Southern hybridisation experiments. Thus GL enzyme activity can be obtained from fungally infected algae, rather than just from the algae as was originally thought.

25

Of particular interest is the finding that there are two natural DNA sequences, each of which codes for an enzyme having GL characteristics. These DNA nucleic acid sequences have been sequenced and they are presented as SEQ. I.D. No. 3 and SEQ. I.D. No. 4 (which are discussed and presented later).

30

An initial enzyme purification can be performed by the method as described by Yu et al (ibid). However, it is preferred that the initial enzyme purification includes the use of a solid support that does not decompose under the purification step. This gel support has the advantage that it is compatible with standard laboratory protein

purification equipment. The details of this preferred purification process are given later on. The purification is terminated by known standard techniques for protein purification. The purity of the enzyme was established using complementary electroforetic techniques.

5

The purified lyase was characterized according to pI, temperature- and pH-optima. In this regard, it was found that the enzyme has the following characteristics: an optimum substrate specificity and a pH optimum at 3.5-7.5 when amylopectin is used; a temperature optimum at 50°C and a pI of 3.9

10

As mentioned above, the enzymes according to the present invention have been determined (partially by amino-acid sequencing techniques) and their amino acid sequences are provided later. Likewise the nucleotide sequences coding for the enzymes according to the present invention (i.e. GL) have been sequenced and the DNA sequences are provided later.

15

The following samples were deposited in accordance with the Budapest Treaty at the recognised depositary The National Collections of Industrial and Marine Bacteria Limited (NCIMB) at 23 St. Machar Drive, Aberdeen, Scotland, United Kingdom, AB2 1RY on 20 June 1994:

20

E. Coli containing plasmid pGL1 (NCIMB 40652) - [ref. DH5alpha-pGL1]; and

E. Coli containing plasmid pGL2 (NCIMB 40653) - [ref. DH5alpha-pGL2].

25

The following sample was accepted as a deposit in accordance with the Budapest Treaty at the recognised depositary The Culture Collection of Algae and Protozoa (CCAP) at Dunstaffnage Marine Laboratory PO Box 3, Oban, Argyll, Scotland, United Kingdom, PA34 4AD on 11 October 1994:

30

Fungally infected *Gracilariopsis lemaneiformis* (CCAP 1373/1) - [ref. GLQ-1 (Qingdao)].

Thus highly preferred embodiments of the present invention include a GL enzyme obtainable from the expression of the GL coding sequences present in plasmids that are the subject of either deposit NCIMB 40652 or deposit NCIMB 40653; and a GL enzyme obtainable from the fungally infected algae that is the subject of deposit
5 CCAP 1373/1.

The present invention will now be described only by way of example.

10 In the following Examples reference is made to the accompanying figures in which:

Figure 1 shows stained fungally infected algae;

Figure 2 shows stained fungally infected algae;

15 Figure 3 shows sections of fungal hypha;

Figure 4 shows sections of fungally infected algae;

20 Figure 5 shows a section of fungally infected algae;

Figure 6 shows a plasmid map of pGL1;

Figure 7 shows a plasmid map of pGL2;

25 Figure 8 shows the amino acid sequence represented as SEQ. I.D. No.3 showing positions of the peptide fragments that were sequenced;

Figure 9 shows the alignment of SEQ. I.D. No. 1 with SEQ. I.D. No.2;

30 Figure 10 is a microphotograph.

In more detail, Figure 1 shows Calcoflour White stainings revealing fungi in upper part and lower part of *Gracilariopsis lemaneiformis* (108x and 294x).

Figure 2 shows PAS/Anilinblue Black staining of *Gracilariopsis lemaneiformis* with fungi. The fungi have a significant higher content of carbohydrates.

Figure 3 shows a micrograph showing longitudinal and grazing sections of two thin-walled fungal hypha (f) growing between thick walls (w) of algal cells. Note thylacoid membranes in the algal chloroplast (arrows).

Figure 4 shows the antisense detections with clone 2 probe (upper row) appear to be restricted to the fungi illustrated by Calcoflour White staining of the succeeding section (lower row) (46x and 108x).

Figure 5 shows intense antisense detections with clone 2 probe are found over the fungi in *Gracilariopsis lemaneiformis* (294x).

Figure 6 shows a map of plasmid pGL1 - which is a pBluescript II KS containing a 3.8 kb fragment isolated from a genomic library constructed from fungal infected *Gracilariopsis lemaneiformis*. The fragment contains a gene coding for alpha-1,4-glucan lyase.

Figure 7 shows a map of plasmid pGL2 - which is a pBluescript II SK containing a 3.6 kb fragment isolated from a genomic library constructed from fungal infected *Gracilariopsis lemaneiformis*. The fragment contains a gene coding for alpha-1,4-glucan lyase.

Figure 9 shows the alignment of SEQ. I.D. No. 1 (GL1) with SEQ. I.D. No.2 (GL2). The total number of residues for GL1 is 1088; and the total number of residues for GL2 is 1091. In making the comparison, a structure-genetic matrix was used (Open gap cost: 10; Unit gap cost: 2). In Figure 9 the character to show that two aligned residues are identical is ':'; and the character to show that two aligned

residues are similar is '. '. Amino acids said to be 'similar' are: A,S,T; D,E; N,Q; R,K; I,L,M,V; F,Y,W. Overall there is an identity of 845 amino acids (i.e. 77.67%); a similarity of 60 amino acids (5.51%). The number of gaps inserted in GL1 are 3 and the number of gaps inserted in GL2 are 2.

5

Figure 10 is a microphotograph of a fungal hypha (f) growing between the algal walls (w). Note grains of floridean starch (s) and thylakoids (arrows) in the algal cell.

10

The following sequence information was used to generate primers for the PCR reactions mentioned below and to check the amino acid sequence generated by the respective nucleotide sequences.

Amino acid sequence assembled from peptides from fungus infected *Gracilariopsis lemaneiformis*

15

Tyr Arg Trp Gln Glu Val Leu Tyr Thr Ala Met Tyr Gln Asn Ala
Ala Phe Gly Lys Pro Ile Ile Lys Ala Ala Ser Met Tyr Asn Asn
Asp Ser Asn Val Arg Arg Ala Gln Asn Asp His Phe Leu Leu Gly
Gly His Asp Gly Tyr Arg Ile Leu Cys Ala Pro Val Val Trp Glu
Asn Ser Thr Glu Arg Glu Leu Tyr Leu Pro Val Leu Thr Gln Trp
Tyr Lys Phe Gly Pro Asp Phe Asp Thr Lys Pro Leu Glu Gly Ala

20

The Amino acid sequence (27-34) used to generate primer A and B (Met Tyr Asn Asn Asp Ser Asn Val)

25

Primer A

ATG TA(TC) AA(CT) AA(CT) GA(CT) TC(GATC) AA(CT) GT 128 mix

Primer B

30

ATG TA(TC) AA(CT) AA(CT) GA(CT) AG(CT) AA(CT) GT 64 mix

The Amino acid sequence (45-50) used to generate primer C (Gly Gly His Asp Gly Tyr)

Primer C

5 TA (GATC)CC (GA)TC (GA)TG (GATC)CC (GATC)CC 256 mix

[The sequence corresponds to the complementary strand.]

The Amino acid sequence (74-79) used to generate primer E (Gln Trp Tyr Lys Phe Gly)

10

Primer E

GG(GATC) CC(GA) AA(CT) TT(GA) TAC CA(CT) TG 64 mix

[The sequence corresponds to the complementary strand.]

15 The Amino acid sequence (1-6) used to generate primer F1 and F2 (Tyr Arg Trp Gln Glu Val)

Primer F1

TA(TC) CG(GATC) TGG CA(GA) GA(GA) GT 32 mix

20

Primer F2

TA(TC) AG(GA) TGG CA(GA) GA(GA) GT 16 mix

The sequence obtained from the first PCR amplification (clone 1)

25

ATGTACAACA ACGACTCGAA CGTTCGCAGG GCGCAGAACG ATCATTTCCT
TCTTGCGGCGC CACGACGGTT A

Met Tyr Asn Asn Asp Ser Asn Val Arg Arg Ala Gln Asn Asp His Phe Leu Leu Gly
30 Gly His Asp Gly

The sequence obtained from the second PCR amplification (clone 1)

ATGTACAACA ACGACTCGAA CGTTCGCAGG GCGCAGAACG ATCATTTCCT
TCTTGGTGGG CATGATGGAT ATCGCATTCT GTGCGCGCCT GTTGTGTGGG
AGAATTCGAC CGAACGNGAA TTGTACTIONGC CCGTGCTGAC CCAATGGTAC
5 AAATTCGGCC C

Met Tyr Asn Asn Asp Ser Asn Val Arg Arg Ala Gln Asn Asp His Phe Leu Leu Gly
Gly His Asp Gly Tyr Arg Ile Leu Cys Ala Pro Val Val Trp Glu Asn Ser Thr Glu
Arg Glu Leu Tyr Leu Pro Val Leu Thr Gln Trp Tyr Lys Phe Gly Pro

The sequence obtained from the third PCR amplification (clone2)

TACAGGTGGC AGGAGGTGTT GTACACTGCT ATGTACCAGA
ATGCGGCTTT CGGGAAACCG ATTATCAAGG CAGCTTCCAT
15 GTACGACAAC GACAGAAACG TTCGCGGCGC ACAGGATGAC
CACTTCCTTC TCGGCGGACA CGATGGATAT CGTATTTTGT
GTGCACCTGT TGTGTGGGAG AATACAACCA GTCGCGATCT
GTACTTGCCT GTGCTGACCA GTGGTACAAA TTCGGCCC

20 Tyr Arg Trp Gln Glu Val Leu Tyr Thr Ala Met Tyr Gln Asn Ala Ala Phe Gly Lys
Pro Ile Ile Lys Ala Ala Ser Met Tyr Asp Asn Asp Arg Asn Val Arg Gly Ala Gln Asp
Asp His Phe Leu Leu Gly Gly His Asp Gly Tyr Arg Ile Leu Cys Ala Pro Val Val
Trp Glu Asn Thr Thr Ser Arg Asp Leu Tyr Leu Pro Val Leu Thr Lys Trp Tyr Lys
Phe Gly

1. CYTOLOGICAL INVESTIGATIONS OF GRACILARIOPSIS LEMANEIFORMIS

1.1.1 Detection of fungal infection in *Gracilariopsis lemaneiformis*

Sections of *Gracilariopsis lemaneiformis* collected in China were either hand cut or cut from paraffin embedded material. Sectioned material was carefully investigated by light microscopy. Fungal hyphae were clearly detected in *Gracilariopsis lemaneiformis*.

The thalli of the *Gracilariopsis lemaneiformis* are composed of cells appearing in a highly ordered and almost symmetric manner. The tubular thallus of *G. lemaneiformis* is composed of large, colourless central cells surrounded by elongated, slender, elliptical cells and small, round, red pigmented peripheral cells. All algal cell types are characterized by thick cell walls. Most of the fungal hyphae are found at the interphase between the central layer of large cells and the peripheral layer. These cells can clearly be distinguished from the algae cells as they are long and cylindrical. The growth of the hyphae is observed as irregularities between the highly ordered algae cells. The most frequent orientation of the hypha is along the main axis of the algal thallus. Side branches toward the central and periphery are detected in some cases. The hypha can not be confused with the endo/epiphytic 2nd generation of the algae.

Calcofluor White is known to stain chitin and cellulose containing tissue. The reaction with chitin requires four covalently linked terminal n-acetyl glucosamine residues. It is generally accepted that cellulose is almost restricted to higher plants although it might occur in trace amounts in some algae. It is further known that chitin is absent in *Gracilaria*.

Calcofluor White was found to stain domains corresponding to fungi hypha cell walls in sectioned *Gracilariopsis lemaneiformis* material.

The hypha appear clear white against a faint blue background of *Gracilaria* tissue when observed under u.v. light - see Figure 1. Chitin is the major cell wall component in most fungi but absent in *Gracilaria*. Based upon these observations we conclude that the investigated algae is infected by a fungi. 40% of the lower parts of the investigated *Gracilariopsis lemaneiformis* sections were found to be infected with fungal hyphae. In the algae tips 25% of the investigated *Gracilariopsis lemaneiformis* sections were found to be infected.

Staining of sectioned *Gracilariopsis lemaneiformis* with Periodic acid Schiff (PAS) and Aniline blue black revealed a significantly higher content of carbohydrates within the fungal cells as compared with the algae cells - see Figure 2. Safranin O and Malachit Green showed the same colour reaction of fungi cells as found in higher plants infected with fungi.

An Acridin Orange reaction with sectioned *Gracilariopsis lemaneiformis* showed clearly the irregularly growth of the fungus.

1.1.2 Electron Microscopy

Slides with 15 μm thick sections, where the fungus was detected with Calcofluor White were fixed in 2% OsO_4 , washed in water and dehydrated in dimethoxypropane and absolute alcohol. A drop of a 1:1 mixture of acetone and Spurr resin was placed over each section on the glass slide, and after one hour replaced by a drop of pure resin. A gelatin embedding capsule filled with resin was placed face down over the section and left over night at 4°C. After the polymerization at 55°C for 8 hrs, the thick sections adhering to the resin blocks could be separated from the slide by immersion in liquid nitrogen.

Blocks were trimmed and 100 nm thick sections were cut using a diamond knife on a microtome. The sections were stained in aqueous uranyl acetate and in lead citrate. The sections were examined in an electron microscope at 80 kV.

The investigation confirmed the light microscopical observations and provided further evidence that the lyase producing, chinese strain of *G. lamneiformis* is infected by a fungal parasite or symbiont.

5 Fungal hyphae are build of tubular cells 50 to 100 μm long and only few microns in diameter. The cells are serially arranged with septate walls between the adjacent cells. Ocasional branches are also seen. The hyphae grow between the thick cell walls of algal thallus without penetrating the wall or damaging the cell. Such a symbiotic association, called mycophycobiosis, is known to occur between some filamentous
10 marine fungi and large marine algae (Donk and Bruning, 1992 - Ecology of aquatic fungi in and on algae. In Reisser, W.(ed.): Algae and Symbioses: Plants, Animals, Fungi, Viruses, Interactions Explored. Biopress Ltd.,Bristol.)

15 Examining the microphotograph in Figure 10, several differences between algal and fungal cells can be noticed. In contrast to several μm thick walls of the alga, the fungal walls are only 100-200 nm thick. Plant typical organells as chloroplasts with thyllacoid membranes as well as floridean starch grains can be seen in algal cells, but not in the fungus.

20 Intercellular connections of red algae are characterized by specific structures termed pit plugs, or pit connections. The structures are prominent, electron dense cores and they are important features in algal taxonomy (Pueschel, C.M.: An expanded survey of the ultrastructure of Red algal pit plugs. J. Phycol. 25, 625, (1989)). In our material, such connections were frequently observed in the algal thallus, but never
25 between the cells of the fungus.

1.2 *In situ* Hybridization experiments

30 *In situ* hybridization technique is based upon the principle of hybridization of an antisense ribonucotide sequence to the mRNA. The technique is used to visualize areas in microscopic sections where said mRNA is present. In this particular case the technique is used to localize the enzyme α -1,4-glucan lyase in sections of

Gracilariopsis lemaneiformis.1.2.1 Preparation of ^{35}S labelled probes for *In situ* hybridization

5 A 238 bp PCR fragment from a third PCR amplification - called clone 2 (see above) - was cloned into the pGEM-3Zf(+) Vector (Promega). The transcription of the antisense RNA was driven by the SP6 promotor, and the sense RNA by the T7 promotor. The Ribonuclease protection assay kit (Ambion) was used with the following modifications. The transcripts were run on a 6% sequencing gel to remove
10 the unincorporated nucleotide and eluted with the elution buffer supplied with the T7RNA polymerase in vitro Transcription Kit (Ambion). The antisense transcript contained 23 non-coding nucleotides while the sense contained 39. For hybridization 10^7 cpm/ml of the ^{35}S labelled probe was used.

15 *In situ* hybridisation was performed essentially as described by Langedale et.al.(1988). The hybridization temperature was found to be optimal at 45°C. After washing at 45°C the sections were covered with Kodak K-5 photographic emulsion and left for 3 days at 5°C in dark (Ref: Langedale, J.A., Rothermel, B.A. and
20 Nelson, T. (1988). Genes and development 2: 106-115. Cold Spring Harbour Laboratory).

The *in situ* hybridization experiments with riboprobes against the mRNA of α -1,4-glucan lyase, show strong hybridizations over and around the hypha of the fungus detected in *Gracilariopsis lemaneiformis* - see Figures 4 and 5. This is considered
25 a strong indication that the α -1,4-glucan lyase is produced. A weak random background reactions were detected in the algae tissue of both *Gracilariopsis lemaneiformis*. This reaction was observed both with the sense and the antisense probes. Intense staining over the fungi hypha was only obtained with antisense probes.

30 These results were obtained with standard hybridisation conditions at 45°C in hybridization and washing steps. At 50°C no staining over the fungi was observed,

whereas the background staining remained the same. Raising the temperature to 55°C reduced the background staining with both sense and antisense probes significantly and equally.

5 Based upon the cytological investigations using complementary staining procedures it is concluded that *Gracilariopsis lemaneiformis* is fungus infected. The infections are most pronounced in the lower parts of the algal tissue.

10 In sectioned *Gracilariopsis lemaneiformis* material *in situ* hybridization results clearly indicate that hybridization is restricted to areas where fungal infections are found - see Figure 4. The results indicate that α -1,4-glucan lyase mRNA appears to be restricted to fungus infected areas in *Gracilariopsis lemaneiformis*.

15 Based upon these observations we conclude that α -1,4-glucan lyase activity is detected in fungally infected *Gracilariopsis lemaneiformis*.

2. ENZYME PURIFICATION AND CHARACTERIZATION

20 Purification of α -1,4-glucan lyase from fungal infected *Gracilariopsis lemaneiformis* material was performed as follows.

2.1 Materials and Methods

25 The algae were harvested by filtration and washed with 0.9% NaCl. The cells were broken by homogenization followed by sonication on ice for 6x3 min in 50 mM citrate-NaOH pH 6.2 (Buffer A). Cell debris were removed by centrifugation at 25,000xg for 40 min. The supernatant obtained at this procedure was regarded as cell-free extract and was used for activity staining and Western blotting after separation on 8-25% gradient gels.

2.2 Separation by β -cyclodextrin Sepharose gel

The cell-free extract was applied directly to a β -cyclodextrin Sepharose gel 4B column (2.6 x 18 cm) pre equilibrated with Buffer A. The column was washed with 3 volumes of Buffer A and 2 volumes of Buffer A containing 1 M NaCl. α -1,4-glucan lyase was eluted with 2 % dextrans in Buffer A. Active fractions were pooled and the buffer changed to 20 mM Bis-tris propane-HCl (pH 7.0, Buffer B).

Active fractions were applied onto a Mono Q HR 5/5 column pre-equilibrated with Buffer B. The fungal lyase was eluted with Buffer B in a linear gradient of 0.3 M NaCl.

The lyase preparation obtained after β -cyclodextrin Sepharose chromatography was alternatively concentrated to 150 μ l and applied on a Superose 12 column operated under FPLC conditions.

2.3 Assay for α -1,4-glucan lyase activity and conditions for determination of substrate specificity, pH and temperature optimum

The reaction mixture for the assay of the α -1,4-glucan lyase activity contained 10 mg ml⁻¹ amylopectin and 25 mM Mes-NaOH (pH 6.0). The reaction was carried out at 30°C for 30 min and stopped by the addition of 3,5-dinitrosalicylic acid reagent. Optical density at 550nm was measured after standing at room temperature for 10 min.

3. AMINO ACID SEQUENCING OF THE α -1,4-GLUCAN LYASE FROM FUNGUS INFECTED GRACILARIOPSIS LEMANEIFORMIS

3.1 Amino acid sequencing of the lyases

5 The lyases were digested with either endoproteinase Arg-C from *Clostridium histolyticum* or endoproteinase Lys-C from *Lysobacter enzymogenes*, both sequencing grade purchased from Boehringer Mannheim, Germany. For digestion with endoproteinase Arg-C, freeze dried lyase (0.1 mg) was dissolved in 50 μ l 10 M urea, 10 50 mM methylamine, 0.1 M Tris-HCl, pH 7.6. After overlay with N₂ and addition of 10 μ l of 50 mM DTT and 5 mM EDTA the protein was denatured and reduced for 10 min at 50°C under N₂. Subsequently, 1 μ g of endoproteinase Arg-C in 10 μ l of 50 mM Tris-HCl, pH 8.0 was added, N₂ was overlayed and the digestion was carried out for 6h at 37°C. For subsequent cysteine derivatization, 12.5 μ l 100 mM iodoaceta- 15 mide was added and the solution was incubated for 15 min at RT in the dark under N₂.

For digestion with endoproteinase Lys-C, freeze dried lyase (0.1 mg) was dissolved in 50 μ l of 8 M urea, 0.4 M NH₄HCO₃, pH 8.4. After overlay with N₂ and addition 20 of 5 μ l of 45 mM DTT, the protein was denatured and reduced for 15 min at 50°C under N₂. After cooling to RT, 5 μ l of 100 mM iodoacetamide was added for the cysteines to be derivatized for 15 min at RT in the dark under N₂.

Subsequently, 90 μ l of water and 5 μ g of endoproteinase Lys-C in 50 μ l of 50 mM 25 tricine and 10 mM EDTA, pH 8.0, was added and the digestion was carried out for 24h at 37°C under N₂.

The resulting peptides were separated by reversed phase HPLC on a VYDAC C18 column (0.46 x 15 cm; 10 μ m; The Separations Group; California) using solvent A: 30 0.1% TFA in water and solvent B: 0.1% TFA in acetonitrile. Selected peptides were rechromatographed on a Develosil C18 column (0.46 x 10 cm; 3 μ m; Dr. Ole Schou, Novo Nordisk, Denmark) using the same solvent system prior to sequencing on an

Applied Biosystems 476A sequencer using pulsed-liquid fast cycles.

The amino acid sequence information from the enzyme derived from fungus infected *Gracilariopsis lemaneiformis* is shown below, in particular SEQ. ID. No. 1. and SEQ. ID. No. 2.

SEQ. I.D. No. 1 has:

Number of residues : 1088.

Amino acid composition (including the signal sequence)

=====

61 Ala	15 Cys	19 His	34 Met	78 Thr
51 Arg	42 Gln	43 Ile	53 Phe	24 Trp
88 Asn	53 Glu	63 Leu	51 Pro	58 Tyr
79 Asp	100 Gly	37 Lys	62 Ser	77 Val

SEQ. I.D. No. 2 has:

Number of residues : 1091.

Amino acid composition (including the signal sequence)

=====

58 Ala	16 Cys	14 His	34 Met	68 Thr
57 Arg	40 Gln	44 Ile	56 Phe	23 Trp
84 Asn	47 Glu	69 Leu	51 Pro	61 Tyr
81 Asp	102 Gly	50 Lys	60 Ser	76 Val

3.2 N-TERMINAL ANALYSIS

Studies showed that the N-terminal sequence of native glucan lyase 1 was blocked. Deblocking was achieved by treating glucan lyase 1 blotted onto a PVDF membrane with anhydrous TFA for 30 min at 40°C essentially as described by LeGendre et al. (1993) [Purification of proteins and peptides by SDS-PAGE; In: Matsudaira, P. (ed.) A practical guide to protein and peptide purification for microsequencing, 2nd edition; Academic Press Inc., San Diego; pp. 74-101.]. The sequence obtained was

TALSDKQTA, which matches the sequence (sequence position from 51 to 59 of SEQ. I.D. No.1) derived from the clone for glucan lyase 1 and indicates N-acetylthreonine as N-terminal residue of glucan lyase 1. Sequence position 1 to 50 of SEQ. I.D. No. 1 represents a signal sequence.

5

4. DNA SEQUENCING OF GENES CODING FOR THE α -1,4-GLUCAN LYASE FROM FUNGUS INFECTED *GRACILARIOPSIS LEMANEIFORMIS*

4.1 METHODS FOR MOLECULAR BIOLOGY

10

DNA was isolated as described by Saunders (1993) with the following modification: The polysaccharides were removed from the DNA by ELUTIP-d (Schleicher & Schuell) purification instead of gel purification. (Ref:Saunders, G.W. (1993). Gel purification of red algal genomic DNA: An inexpensive and rapid method for the isolation of PCR-friendly DNA. Journal of phycology 29(2): 251-254 and Schleicher & Schuell: ELUTIP-d. Rapid Method for Purification and Concentration of DNA.)

15

4.2 PCR

20

The preparation of the relevant DNA molecule was done by use of the Gene Amp DNA Amplification Kit (Perkin Elmer Cetus, USA) and in accordance with the manufactures instructions except that the Taq polymerase was added later (see PCR cycles) and the temperature cycling was changed to the following:

PCR cycles:

25	no of cycles	C	time (min.)
	1	98	5
		60	5
	addition of Taq polymerase and oil		
30	35	94	1
		47	2
		72	3
	1	72	20

4.3 CLONING OF PCR FRAGMENTS

PCR fragments were cloned into pT7Blue (from Novagen) following the instructions of the supplier.

4.4 DNA SEQUENCING

Double stranded DNA was sequenced essentially according to the dideoxy method of Sanger et al. (1979) using the Auto Read Sequencing Kit (Pharmacia) and the Pharmacia LKB A.L.F.DNA sequencer. (Ref: Sanger, F., Nicklen, S. and Coulson, A.R.(1979). DNA sequencing with chain-determinating inhibitors. Proc. Natl. Acad. Sci. USA 74: 5463-5467.).

The sequences are shown as SEQ. I.D.No.s 3 and 4, wherein

SEQ. I.D. No. 3 has:

Total number of bases is: 3267.

DNA sequence composition: 850 A; 761 C; 871 G; 785 T

SEQ. I.D. No. 4 has:

Total number of bases is: 3276.

DNA sequence composition: 889 A; 702 C; 856 G; 829 T

4.5 SCREENING OF THE LIBRARY

Screening of the Lambda Zap library obtained from Stratagene, was performed in accordance with the manufacturer's instructions except that the prehybridization and hybridization was performed in 2xSSC, 0.1% SDS, 10xDenhardt's and 100µg/ml denatured salmon sperm DNA. To the hybridization solution a 32P-labeled denatured probe was added. Hybridization was performed over night at 55°C. The filters were washed twice in 2xSSC, 0.1% SDS and twice in 1xSSC, 0.1% SDS.

4.6 PROBE

The cloned PCR fragments were isolated from the pT7 blue vector by digestion with appropriate restriction enzymes. The fragments were separated from the vector by agarose gel electrophoresis and the fragments were purified from the agarose by Agarase (Boehringer Mannheim). As the fragments were only 90-240 bp long the isolated fragments were exposed to a ligation reaction before labelling with ^{32}P -dCTP using either Prime-It random primer kit (Stratagene) or Ready to Go DNA labelling kit (Pharmacia).

4.7 RESULTS

4.7.1 Generation of PCR DNA fragments coding for α -1,4-glucan lyase.

The amino acid sequences of three overlapping tryptic peptides from α -1,4-glucan lyase were used to generate mixed oligonucleotides, which could be used as PCR primers (see the sequences given above).

In the first PCR amplification primers A/B (see above) were used as upstream primers and primer C (see above) was used as downstream primer. The size of the expected PCR product was 71 base pairs.

In the second PCR amplification primers A/B were used as upstream primers and E was used as downstream primer. The size of the expected PCR product was 161 base pairs.

In the third PCR amplification primers F1 (see above) and F2 (see above) were used as upstream primers and E was used as downstream primer. The size of the expected PCR product was 238 base pairs. The PCR products were analysed on a 2% LMT agarose gel and fragments of the expected sizes were cut out from the gel and treated with Agarase (Boehringer Mannheim) and cloned into the pT7blue Vector (Novagen) and sequenced.

The cloned fragments from the first and second PCR amplification coded for amino acids corresponding to the sequenced peptides (see above). The clone from the third amplification (see above) was only about 87% homologous to the sequenced peptides.

5 4.7.2 Screening of the genomic library with the cloned PCR fragments.

Screening of the library with the above-mentioned clones gave two clones. One clone contained the nucleotide sequence of SEQ I.D. No. 4 (gene 2). The other clone contained some of the sequence of SEQ I.D. No.3 (from base pair 1065 downwards)
10 (gene 1).

The 5' end of SEQ. I.D. No. 3 (i.e. from base pair 1064 upwards) was obtained by the RACE (rapid amplification of cDNA ends) procedure (Michael, A.F., Michael, K.D. & Martin, G.R.(1988). Proc..Natl.Acad.Sci.USA 85:8998-99002.) using the
15 5' race system from Gibco BRL. Total RNA was isolated according to Collinge et al.(Collinge, D.B., Milligan D.E., Dow, J.M., Scofield, G.& Daniels, M.J.(1987). Plant Mol Biol 8: 405-414). The 5' race was done according to the protocol of the manufacturer, using 1µg of total RNA. The PCR product from the second
20 ammplification was cloned into pT7blue vector from Novagen according to the protocol of the manufacturer. Three independent PCR clones were sequenced to compensate for PCR errors.

An additional PCR was performed to supplement the clone just described with XbaI and NdeI restriction sites immediately in front of the ATG start codon using the
25 following oligonucleotide as an upstream primer:
GCTCTAGAGCATGTTTTCAACCCTTGCG and a primer containing the
complement sequence of bp 1573-1593 in sequence GL1 (i.e. SEQ. I.D. No. 3) was used as a downstream primer.

30 The complete sequence for gene 1 (i.e. SEQ. I.D. No. 3) was generated by cloning the 3' end of the gene as a BamHI-HindIII fragment from the genomic clone into the pBluescript II KS+ vector from Stratagene and additionally cloning the PCR

generated 5' end of the gene as a XbaI-BamHI fragment in front of the 3' end.

Gene 2 was cloned as a HindIII blunt ended fragment into the EcoRV site of pBluescript II SK+ vector from Stratagene. A part of the 3' untranslated sequence was removed by a SacI digestion, followed by religation. HindIII and HpaI restriction sites were introduced immediately in front of the start ATG by digestion with HindIII and NarI and religation in the presence of the following annealed oligonucleotides

AGCTTGTTAACATGTATCCAACCCTCACCTTCGTGG
ACAATTGTACATAGGTTGGGAGTGGAAGCACCGC

No introns were found in the clones sequenced.

The clone 1 type (SEQ.ID.No.3) can be aligned with all ten peptide sequences (see Figure 8) showing 100% identity. Alignment of the two protein sequences encoded by the genes isolated from the fungal infected algae *Gracilariopsis lemaneiformis* shows about 78% identity, indicating that both genes are coding for a α -1.4-glucan lyase.

5. EXPRESSION OF THE GL GENE IN MICRO-ORGANISMS

(E.G. ANALYSES OF *PICHIA* LYASE TRANSFORMANTS AND *ASPERGILLUS* LYASE TRANSFORMANTS)

The DNA sequence encoding the GL was introduced into microorganisms to produce an enzyme with high specific activity and in large quantities.

In this regard, gene 1 (i.e. SEQ. I.D. No. 3) was cloned as a NotI-HindIII blunt ended (using the DNA blunting kit from Amersham International) fragment into the *Pichia* expression vector pHIL-D2 (containing the AOX1 promoter) digested with EcoRI and blunt ended (using the DNA blunting kit from Amersham International) for expression in *Pichia pastoris* (according to the protocol stated in the *Pichia*

Expression Kit supplied by Invitrogen).

In another embodiment, the gene 1 (i.e. SEQ. I.D. No. 3) was cloned as a NotI-HindIII blunt ended fragment (using the DNA blunting kit from Amersham International) into the *Aspergillus* expression vector pBARMTE1 (containing the methyl tryptophan resistance promoter from *Neurospora crassa*) digested with SmaI for expression in *Aspergillus niger* (Pall et al (1993) Fungal Genet Newslett. vol 40 pages 59-62). The protoplasts were prepared according to Daboussi et al (Curr Genet (1989) vol 15 pp 453-456) using lysing enzymes Sigma L-2773 and the lyticase Sigma L-8012. The transformation of the protoplasts was followed according to the protocol stated by Buxton et al (Gene (1985) vol 37 pp 207-214) except that for plating the transformed protoplasts the protocol laid out in Punt et al (Methods in Enzymology (1992) vol 216 pp 447 - 457) was followed but with the use of 0.6% osmotic stabilised top agarose.

The results showed that lyase activity was observed in the transformed *Pichia pastoris* and *Aspergillus niger*.

5.1 GENERAL METHODS

Preparation of cell-free extracts.

The cells were harvested by centrifugation at 9000 rpm for 5 min and washed with 0.9% NaCl and resuspended in the breaking buffer (50mM K-phosphate, pH 7.5 containing 1mM of EDTA, and 5% glycerol). Cells were broken using glass beads and vortex treatment. The breaking buffer contained 1 mM PMSF (protease inhibitor). The lyase extract (supernatant) was obtained after centrifugation at 9000 rpm for 5 min followed by centrifugation at 20,000 xg for 5min.

Assay of lyase activity by alkaline 3,5-dinitrosalicylic acid reagent (DNS)

One volume of lyase extract was mixed with an equal volume of 4% amylopectin

solution. The reaction mixture was then incubated at a controlled temperature and samples were removed at specified intervals and analyzed for AF.

The lyase activity was also analyzed using a radioactive method.

5

The reaction mixture contained 10 μ l 14 C-starch solution (1 μ Ci; Sigma Chemicals Co.) and 10 μ l of the lyase extract. The reaction mixture was left at 25°C overnight and was then analyzed in the usual TLC system. The radioactive AF produced was detected using an Instant Imager (Pachard Instrument Co., Inc., Meriden, CT).

10

Electrophoresis and Western blotting

SDS-PAGE was performed using 8-25% gradient gels and the PhastSystem (Pharmacia). Western blottings was also run on a Semidry transfer unit of the PhastSystem.

15

Primary antibodies raised against the lyase purified from the red seaweed collected at Qingdao (China) were used in a dilution of 1:100. Pig antirabbit IgG conjugated to alkaline phosphatase (Dako A/S, Glostrup, Denmark) were used as secondary antibodies and used in a dilution of 1:1000.

20

Part I, Analysis of the Pichia transformants containing the above mentioned construct

25

Results:

1. Lyase activity was determined 5 days after induction (according to the manual) and proved the activity to be intracellular for all samples in the B series.

Samples of B series:	11	12	13	15	26	27	28	29	30
----------------------	----	----	----	----	----	----	----	----	----

Specific activity:	139	81	122	192	151	253	199	198	150
--------------------	-----	----	-----	-----	-----	-----	-----	-----	-----

*Specific activity is defined as nmol AF released per min per mg protein in a reaction mixture containing 2% (w/v) of glycogen, 1% (w/v) glycerol in 10 mM potassium phosphate buffer (pH 7.5). The reaction temperature was 45°C; the reaction time was 60 min.

A time course of sample B27 is as follows. The data are also presented in Figure 1.

Time (min)	0	10	20	30	40	50	60
------------	---	----	----	----	----	----	----

Spec. act.	0	18	54	90	147	179	253
------------	---	----	----	----	-----	-----	-----

Assay conditions were as above except that the time was varied.

2. Western-blotting analysis.

The CFE of all samples showed bands with a molecular weight corresponding to the native lyase.

MC-Lyase expressed intracellularly in *Pichia pastoris*

Names of culture	Specific activity*
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A18	10
-----	----

A20	32
-----	----

A21	8
-----	---

A22	8
-----	---

A24	6
-----	---

Part II, The *Aspergillus* transformants**Results**

I. Lyase activity was determined after 5 days incubation(minimal medium containing 0.2% casein enzymatic hydrolysate analysis by the alkaline 3,5-dinitrosalicylic acid reagent

1). Lyase activity analysis of the culture medium

Among 35 cultures grown with 0.2% amylopectin included in the culture medium, AF was only detectable in two cultures. The culture medium of 5.4+ and 5.9+ contained 0.13 g AF/liter and 0.44 g/liter, respectively. The result indicated that active lyase had been secreted from the cells. Lyase activity was also measurable in the cell-free extract.

2). Lyase activity analysis in cell-free extracts

	Name of the culture	Specific activity*
5	5.4+	51
	5.9+	148
10	5.13	99
	5.15	25
	5.19	37
15		

*The specific activity was defined as nmol of AF produced per min per mg protein at 25°C. + indicates that 0.2% amylopectin was added.

The results show that Gene 1 of GL was expressed intracellular in *A. niger*.

Experiments with transformed *E. coli* (using cloning vectors pQE30 from the Qia express vector kit from Qiagen) showed expression of enzyme that was recognised by anti-body to the enzyme purified from fungally infected *Gracilariopsis lemaneiformis*.

Instead of *Aspergillus niger* as host, other industrial important microorganisms for which good expression systems are known could be used such as: *Aspergillus oryzae*, *Aspergillus sp.*, *Trichoderma sp.*, *Saccharomyces cerevisiae*, *Kluyveromyces sp.*, *Hansenula sp.*, *Pichia sp.*, *Bacillus subtilis*, *B. amyloliquefaciens*, *Bacillus sp.*, *Streptomyces sp.* or *E. coli*.

Other preferred embodiments of the present invention include any one of the following: A transformed host organism having the capability of producing AF as a consequence of the introduction of a DNA sequence as herein described; such a transformed host organism which is a microorganism - preferably wherein the host
5 organism is selected from the group consisting of bacteria, moulds, fungi and yeast; preferably the host organism is selected from the group consisting of *Saccharomyces*, *Kluyveromyces*, *Aspergillus*, *Trichoderma*, *Hansenula*, *Pichia*, *Bacillus*, *Streptomyces*, *Escherichia* such as *Aspergillus oryzae*, *Saccharomyces cerevisiae*, *Bacillus subtilis*, *Bacillus amyloliquefasciens*, *Escherichia coli*.; A method for preparing the sugar 1,5-D-
10 anhydrofructose comprising contacting an alpha 1,4-glucan (e.g. starch) with the enzyme α -1,4-glucan lyase expressed by a transformed host organism comprising a nucleotide sequence encoding the same, preferably wherein the nucleotide sequence is a DNA sequence, preferably wherein the DNA sequence is one of the sequences hereinbefore described; A vector incorporating a nucleotide sequence as hereinbefore
15 described, preferably wherein the vector is a replication vector, preferably wherein the vector is an expression vector containing the nucleotide sequence downstream from a promoter sequence, the vector preferably containing a marker (such as a resistance marker); Cellular organisms, or cell line, transformed with such a vector; A method of producing the product α -1,4-glucan lyase or any nucleotide sequence or
20 part thereof coding for same, which comprises culturing such an organism (or cells from a cell line) transfected with such a vector and recovering the product.

Other modifications of the present invention will be apparent to those skilled in the art without departing from the scope of the invention.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT:

- (A) NAME: DANISCO A/S
- (B) STREET: LANGEBROGADE 1
- (C) CITY: COPENHAGEN
- (D) STATE: COPENHAGEN K
- (E) COUNTRY: DENMARK
- (F) POSTAL CODE (ZIP): DK-1001

(ii) TITLE OF INVENTION: ENZYME

(iii) NUMBER OF SEQUENCES: 20

(iv) COMPUTER READABLE FORM:

- (A) MEDIUM TYPE: Floppy disk
- (B) COMPUTER: IBM PC compatible
- (C) OPERATING SYSTEM: .PC-DOS/MS-DOS
- (D) SOFTWARE: PatentIn Release #1.0, Version #1.25 (EPO)

(v) CURRENT APPLICATION DATA:

APPLICATION NUMBER: WO PCT/EP94/03399

(2) INFORMATION FOR SEQ ID NO: 1:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 1088 amino acids
- (B) TYPE: amino acid
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

```

Met Phe Ser Thr Leu Ala Phe Val Ala Pro Ser Ala Leu Gly Ala Ser
1           5           10           15
Thr Phe Val Gly Ala Glu Val Arg Ser Asn Val Arg Ile His Ser Ala
20           25           30
Phe Pro Ala Val His Thr Ala Thr Arg Lys Thr Asn Arg Leu Asn Val
35           40           45
Ser Met Thr Ala Leu Ser Asp Lys Gln Thr Ala Thr Ala Gly Ser Thr
50           55           60
Asp Asn Pro Asp Gly Ile Asp Tyr Lys Thr Tyr Asp Tyr Val Gly Val
65           70           75           80
Trp Gly Phe Ser Pro Leu Ser Asn Thr Asn Trp Phe Ala Ala Gly Ser
85           90           95

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31

Ser	Thr	Pro	Gly	Gly	Ile	Thr	Asp	Trp	Thr	Ala	Thr	Met	Asn	Val	Asn	100	105	110
Phe	Asp	Arg	Ile	Asp	Asn	Pro	Ser	Ile	Thr	Val	Gln	His	Pro	Val	Gln	115	120	125
Val	Gln	Val	Thr	Ser	Tyr	Asn	Asn	Asn	Ser	Tyr	Arg	Val	Arg	Phe	Asn	130	135	140
Pro	Asp	Gly	Pro	Ile	Arg	Asp	Val	Thr	Arg	Gly	Pro	Ile	Leu	Lys	Gln	145	150	155
Gln	Leu	Asp	Trp	Ile	Arg	Thr	Gln	Glu	Leu	Ser	Glu	Gly	Cys	Asp	Pro	165	170	175
Gly	Met	Thr	Phe	Thr	Ser	Glu	Gly	Phe	Leu	Thr	Phe	Glu	Thr	Lys	Asp	180	185	190
Leu	Ser	Val	Ile	Ile	Tyr	Gly	Asn	Phe	Lys	Thr	Arg	Val	Thr	Arg	Lys	195	200	205
Ser	Asp	Gly	Lys	Val	Ile	Met	Glu	Asn	Asp	Glu	Val	Gly	Thr	Ala	Ser	210	215	220
Ser	Gly	Asn	Lys	Cys	Arg	Gly	Leu	Met	Phe	Val	Asp	Arg	Leu	Tyr	Gly	225	230	235
Asn	Ala	Ile	Ala	Ser	Val	Asn	Lys	Asn	Phe	Arg	Asn	Asp	Ala	Val	Lys	245	250	255
Gln	Glu	Gly	Phe	Tyr	Gly	Ala	Gly	Glu	Val	Asn	Cys	Lys	Tyr	Gln	Asp	260	265	270
Thr	Tyr	Ile	Leu	Glu	Arg	Thr	Gly	Ile	Ala	Met	Thr	Asn	Tyr	Asn	Tyr	275	280	285
Asp	Asn	Leu	Asn	Tyr	Asn	Gln	Trp	Asp	Leu	Arg	Pro	Pro	His	His	Asp	290	295	300
Gly	Ala	Leu	Asn	Pro	Asp	Tyr	Tyr	Ile	Pro	Met	Tyr	Tyr	Ala	Ala	Pro	305	310	315
Trp	Leu	Ile	Val	Asn	Gly	Cys	Ala	Gly	Thr	Ser	Glu	Gln	Tyr	Ser	Tyr	325	330	335
Gly	Trp	Phe	Met	Asp	Asn	Val	Ser	Gln	Ser	Tyr	Met	Asn	Thr	Gly	Asp	340	345	350
Thr	Thr	Trp	Asn	Ser	Gly	Gln	Glu	Asp	Leu	Ala	Tyr	Met	Gly	Ala	Gln	355	360	365
Tyr	Gly	Pro	Phe	Asp	Gln	His	Phe	Val	Tyr	Gly	Ala	Gly	Gly	Gly	Met	370	375	380
Glu	Cys	Val	Val	Thr	Ala	Phe	Ser	Leu	Leu	Gln	Gly	Lys	Glu	Phe	Glu	385	390	395

32

Asn Gln Val Leu Asn Lys Arg Ser Val Met Pro Pro Lys Tyr Val Phe
 405 410 415
 Gly Phe Phe Gln Gly Val Phe Gly Thr Ser Ser Leu Leu Arg Ala His
 420 425 430
 Met Pro Ala Gly Glu Asn Asn Ile Ser Val Glu Glu Ile Val Glu Gly
 435 440 445
 Tyr Gln Asn Asn Asn Phe Pro Phe Glu Gly Leu Ala Val Asp Val Asp
 450 455 460
 Met Gln Asp Asn Leu Arg Val Phe Thr Thr Lys Gly Glu Phe Trp Thr
 465 470 475 480
 Ala Asn Arg Val Gly Thr Gly Gly Asp Pro Asn Asn Arg Ser Val Phe
 485 490 495
 Glu Trp Ala His Asp Lys Gly Leu Val Cys Gln Thr Asn Ile Thr Cys
 500 505 510
 Phe Leu Arg Asn Asp Asn Glu Gly Gln Asp Tyr Glu Val Asn Gln Thr
 515 520 525
 Leu Arg Glu Arg Gln Leu Tyr Thr Lys Asn Asp Ser Leu Thr Gly Thr
 530 535 540
 Asp Phe Gly Met Thr Asp Asp Gly Pro Ser Asp Ala Tyr Ile Gly His
 545 550 555 560
 Leu Asp Tyr Gly Gly Gly Val Glu Cys Asp Ala Leu Phe Pro Asp Trp
 565 570 575
 Gly Arg Pro Asp Val Ala Glu Trp Trp Gly Asn Asn Tyr Lys Lys Leu
 580 585 590
 Phe Ser Ile Gly Leu Asp Phe Val Trp Gln Asp Met Thr Val Pro Ala
 595 600 605
 Met Met Pro His Lys Ile Gly Asp Asp Ile Asn Val Lys Pro Asp Gly
 610 615 620
 Asn Trp Pro Asn Ala Asp Asp Pro Ser Asn Gly Gln Tyr Asn Trp Lys
 625 630 635 640
 Thr Tyr His Pro Gln Val Leu Val Thr Asp Met Arg Tyr Glu Asn His
 645 650 655
 Gly Arg Glu Pro Met Val Thr Gln Arg Asn Ile His Ala Tyr Thr Leu
 660 665 670
 Cys Glu Ser Thr Arg Lys Glu Gly Ile Val Glu Asn Ala Asp Thr Leu
 675 680 685
 Thr Lys Phe Arg Arg Ser Tyr Ile Ile Ser Arg Gly Gly Tyr Ile Gly
 690 695 700

33

Asn Gln His Phe Gly Gly Met Trp Val Gly Asp Asn Ser Thr Thr Ser
 705 710 715 720
 Asn Tyr Ile Gln Met Met Ile Ala Asn Asn Ile Asn Met Asn Met Ser
 725 730 735
 Cys Leu Pro Leu Val Gly Ser Asp Ile Gly Gly Phe Thr Ser Tyr Asp
 740 745 750
 Asn Glu Asn Gln Arg Thr Pro Cys Thr Gly Asp Leu Met Val Arg Tyr
 755 760 765
 Val Gln Ala Gly Cys Leu Leu Pro Trp Phe Arg Asn His Tyr Asp Arg
 770 775 780
 Trp Ile Glu Ser Lys Asp His Gly Lys Asp Tyr Gln Glu Leu Tyr Met
 785 790 795 800
 Tyr Pro Asn Glu Met Asp Thr Leu Arg Lys Phe Val Glu Phe Arg Tyr
 805 810 815
 Arg Trp Gln Glu Val Leu Tyr Thr Ala Met Tyr Gln Asn Ala Ala Phe
 820 825 830
 Gly Lys Pro Ile Ile Lys Ala Ala Ser Met Tyr Asn Asn Asp Ser Asn
 835 840 845
 Val Arg Arg Ala Gln Asn Asp His Phe Leu Leu Gly Gly His Asp Gly
 850 855 860
 Tyr Arg Ile Leu Cys Ala Pro Val Val Trp Glu Asn Ser Thr Glu Arg
 865 870 875 880
 Glu Leu Tyr Leu Pro Val Leu Thr Gln Trp Tyr Lys Phe Gly Pro Asp
 885 890 895
 Phe Asp Thr Lys Pro Leu Glu Gly Ala Met Asn Gly Gly Asp Arg Ile
 900 905 910
 Tyr Asn Tyr Pro Val Pro Gln Ser Glu Ser Pro Ile Phe Val Arg Glu
 915 920 925
 Gly Ala Ile Leu Pro Thr Arg Tyr Thr Leu Asn Gly Glu Asn Lys Ser
 930 935 940
 Leu Asn Thr Tyr Thr Asp Glu Asp Pro Leu Val Phe Glu Val Phe Pro
 945 950 955 960
 Leu Gly Asn Asn Arg Ala Asp Gly Met Cys Tyr Leu Asp Asp Gly Gly
 965 970 975
 Val Thr Thr Asn Ala Glu Asp Asn Gly Lys Phe Ser Val Val Lys Val
 980 985 990
 Ala Ala Glu Gln Asp Gly Gly Thr Glu Thr Ile Thr Phe Thr Asn Asp
 995 1000 1005

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Cys Tyr Glu Tyr Val Phe Gly Gly Pro Phe Tyr Val Arg Val Arg Gly
 1010 1015 1020
 Ala Gln Ser Pro Ser Asn Ile His Val Ser Ser Gly Ala Gly Ser Gln
 1025 1030 1035 1040
 Asp Met Lys Val Ser Ser Ala Thr Ser Arg Ala Ala Leu Phe Asn Asp
 1045 1050 1055
 Gly Glu Asn Gly Asp Phe Trp Val Asp Gln Glu Thr Asp Ser Leu Trp
 1060 1065 1070
 Leu Lys Leu Pro Asn Val Val Leu Pro Asp Ala Val Ile Thr Ile Thr
 1075 1080 1085

(2) INFORMATION FOR SEQ ID NO: 2:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 1091 amino acids
 (B) TYPE: amino acid
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

Met Tyr Pro Thr Leu Thr Phe Val Ala Pro Ser Ala Leu Gly Ala Arg
 1 5 10 15
 Thr Phe Thr Cys Val Gly Ile Phe Arg Ser His Ile Leu Ile His Ser
 20 25 30
 Val Val Pro Ala Val Arg Leu Ala Val Arg Lys Ser Asn Arg Leu Asn
 35 40 45
 Val Ser Met Ser Ala Leu Phe Asp Lys Pro Thr Ala Val Thr Gly Gly
 50 55 60
 Lys Asp Asn Pro Asp Asn Ile Asn Tyr Thr Thr Tyr Asp Tyr Val Pro
 65 70 75 80
 Val Trp Arg Phe Asp Pro Leu Ser Asn Thr Asn Trp Phe Ala Ala Gly
 85 90 95
 Ser Ser Thr Pro Gly Asp Ile Asp Asp Trp Thr Ala Thr Met Asn Val
 100 105 110
 Asn Phe Asp Arg Ile Asp Asn Pro Ser Phe Thr Leu Glu Lys Pro Val
 115 120 125
 Gln Val Gln Val Thr Ser Tyr Lys Asn Asn Cys Phe Arg Val Arg Phe
 130 135 140

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Asn Pro Asp Gly Pro Ile Arg Asp Val Asp Arg Gly Pro Ile Leu Gln
 145 150 155 160
 Gln Gln Leu Asn Trp Ile Arg Lys Gln Glu Gln Ser Lys Gly Phe Asp
 165 170 175
 Pro Lys Met Gly Phe Thr Lys Glu Gly Phe Leu Lys Phe Glu Thr Lys
 180 185 190
 Asp Leu Asn Val Ile Ile Tyr Gly Asn Phe Lys Thr Arg Val Thr Arg
 195 200 205
 Lys Arg Asp Gly Lys Gly Ile Met Glu Asn Asn Glu Val Pro Ala Gly
 210 215 220
 Ser Leu Gly Asn Lys Cys Arg Gly Leu Met Phe Val Asp Arg Leu Tyr
 225 230 235 240
 Gly Thr Ala Ile Ala Ser Val Asn Glu Asn Tyr Arg Asn Asp Pro Asp
 245 250 255
 Arg Lys Glu Gly Phe Tyr Gly Ala Gly Glu Val Asn Cys Glu Phe Trp
 260 265 270
 Asp Ser Glu Gln Asn Arg Asn Lys Tyr Ile Leu Glu Arg Thr Gly Ile
 275 280 285
 Ala Met Thr Asn Tyr Asn Tyr Asp Asn Tyr Asn Tyr Asn Gln Ser Asp
 290 295 300
 Leu Ile Ala Pro Gly Tyr Pro Ser Asp Pro Asn Phe Tyr Ile Pro Met
 305 310 315 320
 Tyr Phe Ala Ala Pro Trp Val Val Val Lys Gly Cys Ser Gly Asn Ser
 325 330 335
 Asp Glu Gln Tyr Ser Tyr Gly Trp Phe Met Asp Asn Val Ser Gln Thr
 340 345 350
 Tyr Met Asn Thr Gly Gly Thr Ser Trp Asn Cys Gly Glu Glu Asn Leu
 355 360 365
 Ala Tyr Met Gly Ala Gln Cys Gly Pro Phe Asp Gln His Phe Val Tyr
 370 375 380
 Gly Asp Gly Asp Gly Leu Glu Asp Val Val Gln Ala Phe Ser Leu Leu
 385 390 395 400
 Gln Gly Lys Glu Phe Glu Asn Gln Val Leu Asn Lys Arg Ala Val Met
 405 410 415
 Pro Pro Lys Tyr Val Phe Gly Tyr Phe Gln Gly Val Phe Gly Ile Ala
 420 425 430
 Ser Leu Leu Arg Glu Gln Arg Pro Glu Gly Gly Asn Asn Ile Ser Val
 435 440 445

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Gln Glu Ile Val Glu Gly Tyr Gln Ser Asn Asn Phe Pro Leu Glu Gly
 450 455 460
 Leu Ala Val Asp Val Asp Met Gln Gln Asp Leu Arg Val Phe Thr Thr
 465 470 475 480
 Lys Ile Glu Phe Trp Thr Ala Asn Lys Val Gly Thr Gly Gly Asp Ser
 485 490 495
 Asn Asn Lys Ser Val Phe Glu Trp Ala His Asp Lys Gly Leu Val Cys
 500 505 510
 Gln Thr Asn Val Thr Cys Phe Leu Arg Asn Asp Asn Gly Gly Ala Asp
 515 520 525
 Tyr Glu Val Asn Gln Thr Leu Arg Glu Lys Gly Leu Tyr Thr Lys Asn
 530 535 540
 Asp Ser Leu Thr Asn Thr Asn Phe Gly Thr Thr Asn Asp Gly Pro Ser
 545 550 555 560
 Asp Ala Tyr Ile Gly His Leu Asp Tyr Gly Gly Gly Gly Asn Cys Asp
 565 570 575
 Ala Leu Phe Pro Asp Trp Gly Arg Pro Gly Val Ala Glu Trp Trp Gly
 580 585 590
 Asp Asn Tyr Ser Lys Leu Phe Lys Ile Gly Leu Asp Phe Val Trp Gln
 595 600 605
 Asp Met Thr Val Pro Ala Met Met Pro His Lys Val Gly Asp Ala Val
 610 615 620
 Asp Thr Arg Ser Pro Tyr Gly Trp Pro Asn Glu Asn Asp Pro Ser Asn
 625 630 635 640
 Gly Arg Tyr Asn Trp Lys Ser Tyr His Pro Gln Val Leu Val Thr Asp
 645 650 655
 Met Arg Tyr Glu Asn His Gly Arg Glu Pro Met Phe Thr Gln Arg Asn
 660 665 670
 Met His Ala Tyr Thr Leu Cys Glu Ser Thr Arg Lys Glu Gly Ile Val
 675 680 685
 Ala Asn Ala Asp Thr Leu Thr Lys Phe Arg Arg Ser Tyr Ile Ile Ser
 690 695 700
 Arg Gly Gly Tyr Ile Gly Asn Gln His Phe Gly Gly Met Trp Val Gly
 705 710 715 720
 Asp Asn Ser Ser Ser Gln Arg Tyr Leu Gln Met Met Ile Ala Asn Ile
 725 730 735
 Val Asn Met Asn Met Ser Cys Leu Pro Leu Val Gly Ser Asp Ile Gly
 740 745 750

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Gly Phe Thr Ser Tyr Asp Gly Arg Asn Val Cys Pro Gly Asp Leu Met
 755 760 765
 Val Arg Phe Val Gln Ala Gly Cys Leu Leu Pro Trp Phe Arg Asn His
 770 775 780
 Tyr Gly Arg Leu Val Glu Gly Lys Gln Glu Gly Lys Tyr Tyr Gln Glu
 785 790 795 800
 Leu Tyr Met Tyr Lys Asp Glu Met Ala Thr Leu Arg Lys Phe Ile Glu
 805 810 815
 Phe Arg Tyr Arg Trp Gln Glu Val Leu Tyr Thr Ala Met Tyr Gln Asn
 820 825 830
 Ala Ala Phe Gly Lys Pro Ile Ile Lys Ala Ala Ser Met Tyr Asp Asn
 835 840 845
 Asp Arg Asn Val Arg Gly Ala Gln Asp Asp His Phe Leu Leu Gly Gly
 850 855 860
 His Asp Gly Tyr Arg Ile Leu Cys Ala Pro Val Val Trp Glu Asn Thr
 865 870 875 880
 Thr Ser Arg Asp Leu Tyr Leu Pro Val Leu Thr Lys Trp Tyr Lys Phe
 885 890 895
 Gly Pro Asp Tyr Asp Thr Lys Arg Leu Asp Ser Ala Leu Asp Gly Gly
 900 905 910
 Gln Met Ile Lys Asn Tyr Ser Val Pro Gln Ser Asp Ser Pro Ile Phe
 915 920 925
 Val Arg Glu Gly Ala Ile Leu Pro Thr Arg Tyr Thr Leu Asp Gly Ser
 930 935 940
 Asn Lys Ser Met Asn Thr Tyr Thr Asp Lys Asp Pro Leu Val Phe Glu
 945 950 955 960
 Val Phe Pro Leu Gly Asn Asn Arg Ala Asp Gly Met Cys Tyr Leu Asp
 965 970 975
 Asp Gly Gly Ile Thr Thr Asp Ala Glu Asp His Gly Lys Phe Ser Val
 980 985 990
 Ile Asn Val Glu Ala Leu Arg Lys Gly Val Thr Thr Thr Ile Lys Phe
 995 1000 1005
 Ala Tyr Asp Thr Tyr Gln Tyr Val Phe Asp Gly Pro Phe Tyr Val Arg
 1010 1015 1020
 Ile Arg Asn Leu Thr Thr Ala Ser Lys Ile Asn Val Ser Ser Gly Ala
 1025 1030 1035 1040
 Gly Glu Glu Asp Met Thr Pro Thr Ser Ala Asn Ser Arg Ala Ala Leu
 1045 1050 1055

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Phe Ser Asp Gly Gly Val Gly Glu Tyr Trp Ala Asp Asn Asp Thr Ser
 1060 1065 1070

Ser Leu Trp Met Lys Leu Pro Asn Leu Val Leu Gln Asp Ala Val Ile
 1075 1080 1085

Thr Ile Thr
 1090

(2) INFORMATION FOR SEQ ID NO: 3:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 3267 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: double
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 3:

ATGTTTTCAA CCCTGCGTT TGTCGCACCT AGTGCCTGG GAGCCAGTAC CTTCGTAGGG	60
GCGGAGGTCA GGTCAAATGT TCGTATCCAT TCCGCTTTTC CAGCTGTGCA CACAGCTACT	120
CGCAAAACCA ATCGCCTCAA TGTATCCATG ACCGCATTGT CCGACAAACA AACGGCTACT	180
GCGGGTAGTA CAGACAATCC GGACGGTATC GACTACAAGA CCTACGATTA CGTCGGAGTA	240
TGGGGTTTCA GCCCCCTCTC CAACACGAAC TGGTTTGCTG CCGGCTCTTC TACCCCGGGT	300
GGCATCACTG ATTGGACGGC TACAATGAAT GTCAACTTCG ACCGTATCGA CAATCCGTCC	360
ATCACTGTCC AGCATCCCGT TCAGGTTTCA GTCACGTCAT ACAACAACAA CAGCTACAGG	420
GTTCGCTTCA ACCCTGATGG CCCTATTCGT GATGTGACTC GTGGGCCTAT CCTCAAGCAG	480
CAACTAGATT GGATTGGAAC GCAGGAGCTG TCAGAGGGAT GTGATCCCGG AATGACTTTC	540
ACATCAGAAG GTTCTTGAC TTTTGAGACC AAGGATCTAA GCGTCATCAT CTACGGAAAT	600
TTCAAGACCA GAGTTACGAG AAAGTCTGAC GGCAAGGTCA TCATGGAAAA TGATGAAGTT	660
GGAAGTGCAT CGTCCGGGAA CAAGTGCCGG GGATTGATGT TCGTTGATAG ATTATACGGT	720
AACGCTATCG CTTCCGTCAA CAAGAACTTC CGCAACGACG CGGTCAAGCA GGAGGGATTC	780
TATGGTGCAG GTGAAGTCAA CTGTAAGTAC CAGGACACCT ACATCTTAGA ACGCACTGGA	840
ATCGCCATGA CAAATTACAA CTACGATAAC TTGAACTATA ACCAGTGGGA CCTTAGACCT	900
CCGCATCATG ATGGTGCCCT CAACCCAGAC TATTATATTC CAATGTACTA CGCAGCACCT	960
TGGTTGATCG TTAATGGATG CGCCGGTACT TCGGAGCAGT ACTCGTATGG ATGGTTCATG	1020

GACAATGTCT CTCAATCTTA CATGAATACT GGAGATACTA CCTGGAATTC TGGACAAGAG	1080
GACCTGGCAT ACATGGGCGC GCAGTATGGA CCATTGACC AACATTTTGT TTACGGTGCT	1140
GGGGGTGGGA TGAATGTGT GGTACAGCG TTCTCTCTC TACAAGGCAA GGAGTTCGAG	1200
AACCAAGTTC TCAACAAACG TTCAGTAATG CCTCCGAAAT ACGTCTTTGG TTTCTTCCAG	1260
GGTGTTCG GGAATCTTC CTTGTTGAGA GCGCATATGC CAGCAGGTGA GAACAACATC	1320
TCAGTCGAAG AAATTGTAGA AGGTTATCAA AACAACAATT TCCCTTTCGA GGGGCTCGCT	1380
GTGGACGTGG ATATGCAAGA CAACTTGCGG GTGTTACCA CGAAGGGCGA ATTTTGGACC	1440
GCAAACAGGG TGGGTACTGG CGGGGATCCA AACAACCGAT CGGTTTTTGA ATGGGCACAT	1500
GACAAAGGCC TTGTTTGTCA GACAAATATA ACTTGCTTCC TGAGGAATGA TAACGAGGGG	1560
CAAGACTACG AGGTCAATCA GACGTTAAGG GAGAGGCAGT TGTACACGAA GAACGACTCC	1620
CTGACGGGTA CGGATTTTGG AATGACCGAC GACGGCCCCA GCGATGCGTA CATCGGTCAT	1680
CTGGACTATG GGGGTGGAGT AGAATGTGAT GCACTTTTCC CAGACTGGGG ACGGCCTGAC	1740
GTGGCCGAAT GGTGGGGAAA TAACTATAAG AAAGTTTCA GCATTGGTCT CGACTTCGTC	1800
TGGCAAGACA TGAATGTTCC AGCAATGATG CCGCACAAA TTGGCGATGA CATCAATGTG	1860
AAACCGGATG GGAATTGGCC GAATGCGGAC GATCCGTCCA ATGGACAATA CAACTGGAAG	1920
ACGTACCATC CCCAAGTGCT TGTAAGTATG ATGCGTTATG AGAATCATGG TCGGGAACCG	1980
ATGGTCACTC AACGCAACAT TCATGCGTAT AACTGTGCG AGTCTACTAG GAAGGAAGGG	2040
ATCGTGGAAC ACGCAGACAC TCTAACGAAG TTCCGCCGTA GCTACATTAT CAGTCGTGGT	2100
GGTTACATTG GTAACCAGCA TTTCGGGGGT ATGTGGGTGG GAGACAACTC TACTACATCA	2160
AACTACATCC AAATGATGAT TGCCAACAAT ATTAACATGA ATATGTCTTG CTTGCCTCTC	2220
GTCGGCTCCG ACATTGGAGG ATTCACCTCA TACGACAATG AGAATCAGCG AACGCCGTGT	2280
ACCGGGGACT TGATGGTGAG GTATGTGCAG GCGGGCTGCC TGTTGCCGTG GTTCAGGAAC	2340
CACTATGATA GGTGGATCGA GTCCAAGGAC CACGGAAAGG ACTACCAGGA GCTGTACATG	2400
TATCCGAATG AAATGGATAC GTTGAGGAAG TTCGTTGAAT TCCGTTATCG CTGGCAGGAA	2460
GTGTTGTACA CGGCCATGTA CCAGAATGCG GCTTTCGGAA AGCCGATTAT CAAGGCTGCT	2520
TCGATGTACA ATAACGACTC AAACGTTGCG AGGGCGCAGA ACGATCATTT CCTTCTTGGT	2580
GGACATGATG GATATCGCAT TCTGTGCGCG CCTGTTGTGT GGGAGAATTC GACCGAACGC	2640
GAATTGTACT TGCCCGTGCT GACCCAATGG TACAAATTCG GTCCCGACTT TGACACCAAG	2700

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CCTCTGGAAG GAGCGATGAA CGGAGGGGAC CGAATTTACA ACTACCCTGT ACCGCAAAGT	2760
GAATCACCAA TCTTCGTGAG AGAAGGTGCG ATTCTCCCTA CCCGCTACAC GTTGAACGGT	2820
GAAAACAAAT CATTGAACAC GTACACGGAC GAAGATCCGT TGGTGTTTGA AGTATTCCCC	2880
CTCGGAAACA ACCGTGCCGA CGGTATGTGT TATCTTGATG ATGGCGGTGT GACCACCAAT	2940
GCTGAAGACA ATGGCAAGTT CTCTGTCGTC AAGGTGGCAG CGGAGCAGGA TGGTGGTACG	3000
GAGACGATAA CGTTTACGAA TGATTGCTAT GAGTACGTTT TCGGTGGACC GTTCTACGTT	3060
CGAGTGCGCG GCGCTCAGTC GCCGTCGAAC ATCCACGTGT CTTCTGGAGC GGGTTCTCAG	3120
GACATGAAGG TGAGCTCTGC CACTTCCAGG GCTGCGCTGT TCAATGACGG GGAGAACGGT	3180
GATTTCTGGG TTGACCAGGA GACAGATTCT CTGTGGCTGA AGTTGCCCAA CGTTGTTCTC	3240
CCGGACGCTG TGATCACAAT TACCTAA	3267

(2) INFORMATION FOR SEQ ID NO: 4:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 3276 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: double
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 4:

ATGTATCCAA CCCTCACCTT CGTGGCGCCT AGTGCCTAG GGGCCAGAAC TTTCACGTGT	60
GTGGGCATTT TTAGGTCACA CATTCTTATT CATTGGTTG TTCCAGCGGT GCGTCTAGCT	120
GTGCGCAAAA GCAACCGCCT CAATGTATCC ATGTCCGCTT TGTTGACAA ACCGACTGCT	180
GTTACTGGAG GGAAGGACAA CCCGGACAAT ATCAATTACA CCACTTATGA CTACGTCCCT	240
GTGTGGCGCT TCGACCCCCT CAGCAATACG AACTGGTTTG CTGCCGGATC TTCCACTCCC	300
GGCGATATTG ACGACTGGAC GGCAGCAATG AATGTGAACT TCGACCGTAT CGACAATCCA	360
TCCTTCACTC TCGAGAAACC GGTTCAGGTT CAGGTCACGT CATAAAGAA CAATTGTTTC	420
AGGGTTCGCT TCAACCCTGA TGGTCCTATT CGCGATGTGG ATCGTGGGCC TATCCTCCAG	480
CAGCAACTAA ATTGGATCCG GAAGCAGGAG CAGTCGAAGG GGTTCGATCC TAAGATGGGC	540
TTCACAAAAG AAGGTTTCTT GAAATTTGAG ACCAAGGATC TGAACGTTAT CATATATGGC	600
AATTTTAAGA CTAGAGTTAC GAGGAAGAGG GATGGAAAAG GGATCATGGA GAATAATGAA	660

GTGCCGGCAG GATCGTTAGG GAACAAGTGC CGGGGATTGA TGTTTGTCTGA CAGGTTGTAC	720
GGCACTGCCA TCGCTTCCGT TAATGAAAAT TACCGCAACG ATCCCGACAG GAAAGAGGGG	780
TTCTATGGTG CAGGAGAAGT AAAGTGCAG TTTTGGGACT CCGAACAAAA CAGGAACAAG	840
TACATCTTAG AACGAACTGG AATCGCCATG ACAAATTACA ATTATGACAA CTATAACTAC	900
AACCAGTCAG ATCTTATTGC TCCAGGATAT CCTTCCGACC CGAACTTCTA CATTCCCATG	960
TATTTTGCAG CACCTTGGGT AGTTGTAAAG GGATGCAGTG GCAACAGCGA TGAACAGTAC	1020
TCGTACGGAT GGTATATGGA TAATGTCTCC CAACTTACA TGAATACTGG TGGTACTTCC	1080
TGGAAGTGTG GAGAGGAGAA CTTGGCATACT ATGGGAGCAC AGTGCGGTCC ATTTGACCAA	1140
CATTTTGTGT ATGGTGATGG AGATGGTCTT GAGGATGTTG TCCAAGCGTT CTCTCTTCTG	1200
CAAGGCAAAG AGTTTGAGAA CCAAGTTCTG AACAAACGTG CCGTAATGCC TCCGAAATAT	1260
GTGTTTGGTT ACTTTGAGG AGTCTTTGGG ATTGCTTCCT TGTTGAGAGA GCAAAGACCA	1320
GAGGGTGGTA ATAACATCTC TGTTCAAGAG ATTGTGGAAG GTTACCAAAG CAATAACTTC	1380
CCTTTAGAGG GGTTAGCCGT AGATGTGGAT ATGCAACAAG ATTTGCGCGT GTTCACCACG	1440
AAGATTGAAT TTTGGACGGC AAATAAGGTA GGCACCGGGG GAGACTCGAA TAACAAGTCG	1500
GTGTTTGAAT GGGCACATGA CAAAGGCCTT GTATGTCAGA CGAATGTTAC TTGCTTCTTG	1560
AGAAACGACA ACGGCGGGGC AGATTACGAA GTCAATCAGA CATTGAGGGA GAAGGGTTTG	1620
TACACGAAGA ATGACTCACT GACGAACACT AACTTCGGAA CTACCAACGA CGGGCCGAGC	1680
GATGCGTACA TTGGACATCT GGAATATGGT GGCAGGAGGA ATTGTGATGC ACTTTTCCCA	1740
GACTGGGGTC GACCGGGTGT GGCTGAATGG TGGGGTGATA ACTACAGCAA GCTCTTCAAA	1800
ATTGGTCTGG ATTTGCTCTG GCAAGACATG ACAGTTCCAG CTATGATGCC ACACAAAGTT	1860
GGCGACGCAG TCGATACGAG ATCACCTTAC GGCTGGCCGA ATGAGAATGA TCCTTCGAAC	1920
GGACGATACA ATTGGAAATC TTACCATCCA CAAGTTCTCG TAACTGATAT GCGATATGAG	1980
AATCATGGAA GGAACCGAT GTTCACTCAA CGCAATATGC ATGCGTACAC ACTCTGTGAA	2040
TCTACGAGGA AGGAAGGGAT TGTTGCAAAT GCAGACACTC TAACGAAGTT CCGCCGCAGT	2100
TATATTATCA GTCGTGGAGG TTACATTGGC AACCAGCATT TTGGAGGAAT GTGGGTGGA	2160
GACAACTCTT CCTCCCAAAG ATACCTCAA ATGATGATCG CGAACATCGT CAACATGAAC	2220
ATGTCTTGCC TTCCACTAGT TGGGTCCGAC ATTGGAGGTT TTAATTGTA TGATGGACGA	2280
AACGTGTGTC CCGGGGATCT AATGGTAAGA TTCGTGCAGG CGGGTTGCTT ACTACCGTGG	2340

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TTCAGAAACC ACTATGGTAG GTTGGTCGAG GGCAAGCAAG AGGGAAAATA CTATCAAGAA	2400
CTGTACATGT ACAAGGACGA GATGGCTACA TTGAGAAAAT TCATTGAATT CCGTTACCGC	2460
TGGCAGGAGG TGTGTACAC TGCTATGTAC CAGAATGCGG CTTTCGGGAA ACCGATTATC	2520
AAGGCAGCTT CCATGTACGA CAACGACAGA AACGTTGCGG GCGCACAGGA TGACCACTTC	2580
CTTCTCGGCG GACACGATGG ATATCGTATT TTGTGTGCAC CTGTTGTGTG GGAGAATACA	2640
ACCAGTCGCG ATCTGTACTT GCCTGTGCTG ACCAAATGGT ACAAATTCGG CCCTGACTAT	2700
GACACCAAGC GCCTGGATTC TGC GTTGGAT GGAGGGCAGA TGATTAAGAA CTATTCTGTG	2760
CCACAAAGCG ACTCTCCGAT ATTTGTGAGG GAAGGAGCTA TTCTCCCTAC CCGCTACACG	2820
TTGGACGGTT CGAACAAGTC AATGAACACG TACACAGACA AAGACCCGTT GGTGTTTGAG	2880
GTATTCCTC TTGGAAACAA CCGTGCCGAC GGTATGTGTT ATCTTGATGA TGGCGGTATT	2940
ACTACAGATG CTGAGGACCA TGGCAAATTC TCTGTTATCA ATGTCGAAGC CTTACGGAAA	3000
GGTGTACGA CGACGATCAA GTTTGCGTAT GACACTTATC AATACGTATT TGATGGTCCA	3060
TTCTACGTTT GAATCCGTAA TCTTACGACT GCATCAAAAA TTAACGTGTC TTCTGGAGCG	3120
GGTGAAGAGG ACATGACACC GACCTCTGCG AACTCGAGGG CAGCTTTGTT CAGTGATGGA	3180
GGTGTGGAG AATACTGGGC TGACAATGAT ACGTCTTCTC TGTGGATGAA GTTGCCAAAC	3240
CTGGTTCTGC AAGACGCTGT GATTACCATT ACGTAG	3276

(2) INFORMATION FOR SEQ ID NO: 5:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 90 amino acids
- (B) TYPE: amino acid
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 5:

Tyr	Arg	Trp	Gln	Glu	Val	Leu	Tyr	Thr	Ala	Met	Tyr	Gln	Asn	Ala	Ala
1				5					10					15	
Phe	Gly	Lys	Pro	Ile	Ile	Lys	Ala	Ala	Ser	Met	Tyr	Asn	Asn	Asp	Ser
			20					25					30		
Asn	Val	Arg	Arg	Ala	Gln	Asn	Asp	His	Phe	Leu	Leu	Gly	Gly	His	Asp
		35					40					45			

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Gly Tyr Arg Ile Leu Cys Ala Pro Val Val Trp Glu Asn Ser Thr Glu
 50 55 60

Arg Glu Leu Tyr Leu Pro Val Leu Thr Gln Trp Tyr Lys Phe Gly Pro
 65 70 75 80

Asp Phe Asp Thr Lys Pro Leu Glu Gly Ala
 85 90

(2) INFORMATION FOR SEQ ID NO: 6:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 23 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(ix) FEATURE:

- (A) NAME/KEY: misc difference
- (B) LOCATION: replace(6, "")
- (D) OTHER INFORMATION: /note= "N is T or C"

(ix) FEATURE:

- (A) NAME/KEY: misc difference
- (B) LOCATION: replace(9, "")
- (D) OTHER INFORMATION: /note= "N is C or T"

(ix) FEATURE:

- (A) NAME/KEY: misc difference
- (B) LOCATION: replace(12, "")
- (D) OTHER INFORMATION: /note= "N is C or T"

(ix) FEATURE:

- (A) NAME/KEY: misc difference
- (B) LOCATION: replace(15, "")
- (D) OTHER INFORMATION: /note= "N is C or T"

(ix) FEATURE:

- (A) NAME/KEY: misc difference
- (B) LOCATION: replace(18, "")
- (D) OTHER INFORMATION: /note= "N is G or A or T or C"

(ix) FEATURE:

- (A) NAME/KEY: misc difference
- (B) LOCATION: replace(21, "")
- (D) OTHER INFORMATION: /note= "N is C or T"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 6:

ATGTANAANA ANGANTCNAAGT

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(2) INFORMATION FOR SEQ ID NO: 7:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 23 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(ix) FEATURE:

- (A) NAME/KEY: misc difference
- (B) LOCATION: replace(6, "")
- (D) OTHER INFORMATION: /note= "N is T or C"

(ix) FEATURE:

- (A) NAME/KEY: misc difference
- (B) LOCATION: replace(9, "")
- (D) OTHER INFORMATION: /note= "N is C or T"

(ix) FEATURE:

- (A) NAME/KEY: misc difference
- (B) LOCATION: replace(12, "")
- (D) OTHER INFORMATION: /note= "N is C or T"

(ix) FEATURE:

- (A) NAME/KEY: misc difference
- (B) LOCATION: replace(15, "")
- (D) OTHER INFORMATION: /note= "N is C or T"

(ix) FEATURE:

- (A) NAME/KEY: misc difference
- (B) LOCATION: replace(18, "")
- (D) OTHER INFORMATION: /note= "N is C or T"

(ix) FEATURE:

- (A) NAME/KEY: misc difference
- (B) LOCATION: replace(21, "")
- (D) OTHER INFORMATION: /note= "N is C or T"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 7:

ATGTANAANA ANGANAGNAA NGT

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(2) INFORMATION FOR SEQ ID NO: 8:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 17 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

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- (ix) FEATURE:
 (A) NAME/KEY: misc_difference
 (B) LOCATION: replace(3, "")
 (D) OTHER INFORMATION: /note= "N is G or A or T or C"
- (ix) FEATURE:
 (A) NAME/KEY: misc_difference
 (B) LOCATION: replace(6, "")
 (D) OTHER INFORMATION: /note= "N is G or A"
- (ix) FEATURE:
 (A) NAME/KEY: misc_difference
 (B) LOCATION: replace(9, "")
 (D) OTHER INFORMATION: /note= "N is G or A"
- (ix) FEATURE:
 (A) NAME/KEY: misc_difference
 (B) LOCATION: replace(12, "")
 (D) OTHER INFORMATION: /note= "N is G or A or T or C"
- (ix) FEATURE:
 (A) NAME/KEY: misc_difference
 (B) LOCATION: replace(15, "")
 (D) OTHER INFORMATION: /note= "N is G or A or T or C"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 8:

TANCCNTCNT GNCCNCC

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(2) INFORMATION FOR SEQ ID NO: 9:

- (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 20 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: DNA (genomic)
- (ix) FEATURE:
 (A) NAME/KEY: misc_difference
 (B) LOCATION: replace(3, "")
 (D) OTHER INFORMATION: /note= "N is G or A or T or C"
- (ix) FEATURE:
 (A) NAME/KEY: misc_difference
 (B) LOCATION: replace(6, "")
 (D) OTHER INFORMATION: /note= "N is G or A"
- (ix) FEATURE:
 (A) NAME/KEY: misc_difference
 (B) LOCATION: replace(9, "")
 (D) OTHER INFORMATION: /note= "N is C or T"

37 / 9

(ix) FEATURE:

- (A) NAME/KEY: misc_difference
- (B) LOCATION: replace(12, "")
- (D) OTHER INFORMATION: /note= "N is G or A"

(ix) FEATURE:

- (A) NAME/KEY: misc_difference
- (B) LOCATION: replace(18, "")
- (D) OTHER INFORMATION: /note= "N is C or T"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 9:

GGNCCNAANT TNTACCANTG

20

(2) INFORMATION FOR SEQ ID NO: 10:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 17 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(ix) FEATURE:

- (A) NAME/KEY: misc_difference
- (B) LOCATION: replace(3, "")
- (D) OTHER INFORMATION: /note= "N is T or C"

(ix) FEATURE:

- (A) NAME/KEY: misc_difference
- (B) LOCATION: replace(6, "")
- (D) OTHER INFORMATION: /note= "N is G or A or T or C"

(ix) FEATURE:

- (A) NAME/KEY: misc_difference
- (B) LOCATION: replace(12, "")
- (D) OTHER INFORMATION: /note= "N is G or A"

(ix) FEATURE:

- (A) NAME/KEY: misc_difference
- (B) LOCATION: replace(15, "")
- (D) OTHER INFORMATION: /note= "N is G or A"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 10:

TANCGNTGGC ANGANGT

17

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(2) INFORMATION FOR SEQ ID NO: 11:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 17 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(ix) FEATURE:

- (A) NAME/KEY: misc difference
- (B) LOCATION: replace(3, "")
- (D) OTHER INFORMATION: /note= "N is T or C"

(ix) FEATURE:

- (A) NAME/KEY: misc difference
- (B) LOCATION: replace(6, "")
- (D) OTHER INFORMATION: /note= "N is G or A"

(ix) FEATURE:

- (A) NAME/KEY: misc difference
- (B) LOCATION: replace(12, "")
- (D) OTHER INFORMATION: /note= "N is G or A"

(ix) FEATURE:

- (A) NAME/KEY: misc difference
- (B) LOCATION: replace(15, "")
- (D) OTHER INFORMATION: /note= "N is G or A"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 11:

TANAGNTGGC ANGANGT

17

(2) INFORMATION FOR SEQ ID NO: 12:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 71 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 12:

ATGTACAACA ACGACTCGAA CGTTCGCAGG GCGCAGAACG ATCATTTCCT TCTTGGCGGC

60

CACGACGGTT A

71

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(2) INFORMATION FOR SEQ ID NO: 13:

- (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 23 amino acids
 (B) TYPE: amino acid
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 13:

Met Tyr Asn Asn Asp Ser Asn Val Arg Arg Ala Gln Asn Asp His Phe
 1 5 10 15
 Leu Leu Gly Gly His Asp Gly
 20

(2) INFORMATION FOR SEQ ID NO: 14:

- (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 160 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 14:

ATGTACAACA ACGACTCGAA CGTTCGCAGG GCGCAGAACG ATCATTTCCT TCTTGGTGGG 60
 CATGATGGAT ATCGCATTCT GTGCGCGCCT GTTGTGTGGG AGAATTCGAC CGAACGGAAT 120
 TGTACTTGCC CGTGCTGACC CAATGGTACA AATTCGGCCC 160

(2) INFORMATION FOR SEQ ID NO: 15:

- (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 54 amino acids
 (B) TYPE: amino acid
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 15:

Met Tyr Asn Asn Asp Ser Asn Val Arg Arg Ala Gln Asn Asp His Phe
 1 5 10 15

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Leu Leu Gly Gly His Asp Gly Tyr Arg Ile Leu Cys Ala Pro Val Val
 20 25 30

Trp Glu Asn Ser Thr Glu Arg Glu Leu Tyr Leu Pro Val Leu Thr Gln
 35 40 45

Trp Tyr Lys Phe Gly Pro
 50

(2) INFORMATION FOR SEQ ID NO: 16:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 238 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 16:

TACAGGTGGC AGGAGGTGTT GTACACTGCT ATGTACCAGA ATGCGGCTTT CGGGAAACCG	60
ATTATCAAGG CAGCTTCCAT GTACGACAAC GACAGAAACG TTCGCGGCGC ACAGGATGAC	120
CACTTCCTTC TCGGCGGACA CGATGGATAT CGTATTTTGT GTGCACCTGT TGTGTGGGAG	180
AATACAACCA GTCGCGATCT GTACTTGCCT GTGCTGACCA GTGGTACAAA TTCGGCCC	238

(2) INFORMATION FOR SEQ ID NO: 17:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 79 amino acids
- (B) TYPE: amino acid
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 17:

Tyr Arg Trp Gln Glu Val Leu Tyr Thr Ala Met Tyr Gln Asn Ala Ala	1 5 10 15
Phe Gly Lys Pro Ile Ile Lys Ala Ala Ser Met Tyr Asp Asn Asp Arg	20 25 30
Asn Val Arg Gly Ala Gln Asp Asp His Phe Leu Leu Gly Gly His Asp	35 40 45
Gly Tyr Arg Ile Leu Cys Ala Pro Val Val Trp Glu Asn Thr Thr Ser	50 55 60

Arg Asp Leu Tyr Leu Pro Val Leu Thr Lys Trp Tyr Lys Phe Gly
 65 70 75

(2) INFORMATION FOR SEQ ID NO: 18:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 28 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 18:

GCTCTAGAGC ATGTTTTCAA CCCTTGCG

28

(2) INFORMATION FOR SEQ ID NO: 19:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 36 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 19:

AGCTTGTTAA CATGTATCCA ACCCTCACCT TCGTGG

36

(2) INFORMATION FOR SEQ ID NO: 20:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 34 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 20:

ACAATTGTAC ATAGGTTGGG AGTGGAAGCA CCGC

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INDICATIONS RELATING TO A DEPOSITED MICROORGANISM

(PCT Rule 13bis)

A. The indications made below relate to the microorganism referred to in the description on page <u>5</u> , line <u>22</u>	
B. IDENTIFICATION OF DEPOSIT <div style="text-align: right;">Further deposits are identified on an additional sheet <input type="checkbox"/></div>	
Name of depositary institution The National Collections of Industrial and Marine Bacteria Limited (NCIMB)	
Address of depositary institution (including postal code and country) 23 St. Machar Drive Aberdeen Scotland AB2 1RY United Kingdom	
Date of deposit 20 JUNE 1994	Accession Number NCIMB 40652
C. ADDITIONAL INDICATIONS (leave blank if not applicable) <div style="text-align: right;">This information is continued on an additional sheet <input type="checkbox"/></div>	
In respect of those designations in which a European patent is sought, and any other designated state having equivalent legislation, a sample of the deposited microorganism will be made available until the publication of the mention of the grant of the European patent or until the date on which the application has been refused or withdrawn or is deemed to be withdrawn, only by the issue of such a sample to an expert nominated by the person requesting the sample. (Rule 28(4) EPC).	
D. DESIGNATED STATES FOR WHICH INDICATIONS ARE MADE (if the indications are not for all designated States)	
E. SEPARATE FURNISHING OF INDICATIONS (leave blank if not applicable)	
The indications listed below will be submitted to the International Bureau later (specify the general nature of the indications e.g., "Accession Number of Deposit")	

<div style="text-align: center;">For receiving Office use only</div> <div style="border: 1px solid black; padding: 5px;"> <input type="checkbox"/> This sheet was received with the international application </div> <div style="border: 1px solid black; padding: 5px; margin-top: 10px;"> Authorized officer </div>	<div style="text-align: center;">For International Bureau use only</div> <div style="border: 1px solid black; padding: 5px;"> <input type="checkbox"/> This sheet was received by the International Bureau on: </div> <div style="border: 1px solid black; padding: 5px; margin-top: 10px;"> Authorized officer </div>
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INDICATIONS RELATING TO A DEPOSITED MICROORGANISM

(PCT Rule 13bis)

A. The indications made below relate to the microorganism referred to in the description on page <u>5</u> , line <u>24</u>	
B. IDENTIFICATION OF DEPOSIT	
Further deposits are identified on an additional sheet <input type="checkbox"/>	
Name of depositary institution The National Collections of Industrial and Marine Bacteria Limited (NCIMB)	
Address of depositary institution (including postal code and country) 23 St. Machar Drive Aberdeen Scotland AB2 1RY United Kingdom	
Date of deposit 20 JUNE 1994	Accession Number NCIMB 40653
C. ADDITIONAL INDICATIONS (leave blank if not applicable)	
This information is continued on an additional sheet <input type="checkbox"/>	
In respect of those designations in which a European patent is sought, and any other designated state having equivalent legislation, a sample of the deposited microorganism will be made available until the publication of the mention of the grant of the European patent or until the date on which the application has been refused or withdrawn or is deemed to be withdrawn, only by the issue of such a sample to an expert nominated by the person requesting the sample. (Rule 28(4) EPC).	
D. DESIGNATED STATES FOR WHICH INDICATIONS ARE MADE (if the indications are not for all designated States)	
E. SEPARATE FURNISHING OF INDICATIONS (leave blank if not applicable)	
The indications listed below will be submitted to the International Bureau later (specify the general nature of the indications e.g., "Accession Number of Deposit")	
For receiving Office use only <input type="checkbox"/> This sheet was received with the international application Authorized officer	For International Bureau use only <input type="checkbox"/> This sheet was received by the International Bureau on: Authorized officer

INDICATIONS RELATING TO A DEPOSITED MICROORGANISM

(PCT Rule 13bis)

A. The indications made below relate to the microorganism referred to in the description on page <u>5</u> , line <u>31</u>	
B. IDENTIFICATION OF DEPOSIT	
Further deposits are identified on an additional sheet <input type="checkbox"/>	
Name of depositary institution Culture Collection of Algae and Protozoa (CCAP)	
Address of depositary institution (including postal code and country) Dunstaffnage Marine Laboratory P.O. Box 3 Oban Argyll PA34 4AD Scotland United Kingdom	
Date of deposit 11 OCTOBER 1994	Accession Number CCAP 1373/1
C. ADDITIONAL INDICATIONS (leave blank if not applicable)	
This information is continued on an additional sheet <input type="checkbox"/>	
In respect of those designations in which a European patent is sought, and any other designated state having equivalent legislation, a sample of the deposited microorganism will be made available until the publication of the mention of the grant of the European patent or until the date on which the application has been refused or withdrawn or is deemed to be withdrawn, only by the issue of such a sample to an expert nominated by the person requesting the sample. (Rule 28(4) EPC).	
D. DESIGNATED STATES FOR WHICH INDICATIONS ARE MADE (if the indications are not for all designated States)	
E. SEPARATE FURNISHING OF INDICATIONS (leave blank if not applicable)	
The indications listed below will be submitted to the International Bureau later (specify the general nature of the indications e.g., "Accession Number of Deposit")	
For receiving Office use only <input type="checkbox"/> This sheet was received with the international application Authorized officer	For International Bureau use only <input type="checkbox"/> This sheet was received by the International Bureau on: Authorized officer

CLAIMS

1. A method of preparing the enzyme α -1,4-glucan lyase (GL) comprising isolating the enzyme from a fungally infected algae.

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2. A method according to claim 1 wherein the enzyme is isolated and/or further purified using a gel that is not degraded by the enzyme.

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3. A method according to claim 2 wherein the gel is based on dextrin or derivatives thereof, preferably a cyclodextrin, more preferably beta-cyclo-dextrin.

4. A GL enzyme prepared by the method according to any one of claims 1 to 3.

15

5. An enzyme comprising the amino acid sequence SEQ. ID. No. 1. or SEQ. ID. No. 2, or any variant thereof.

6. A nucleotide sequence coding for the enzyme α -1,4-glucan lyase.

20

7. A nucleotide sequence according to claim 6 wherein the sequence is a DNA sequence.

25

8. A nucleotide sequence according to claim 7 wherein the DNA sequence comprises a sequence that is the same as, or is complementary to, or has substantial homology with, or contains any suitable codon substitutions for any of those of, SEQ. ID. No. 3 or SEQ. ID. No. 4.

9. A method of preparing the enzyme α -1,4-glucan lyase comprising expressing the nucleotide sequence according to any one of claims 6 to 8.

30

10. A method according to any one of the preceding claims wherein the algae is *Gracilariopsis lemaneiformis*.

11. The use of beta-cyclodextrin to purify an enzyme, preferably GL.

5

12. A nucleotide sequence wherein the DNA sequence comprises a sequence that is the same as, or is complementary to, or has substantial homology with, or contains any suitable codon substitutions for any of those of, SEQ. ID. No. 3 or SEQ. ID. No. 4.

10

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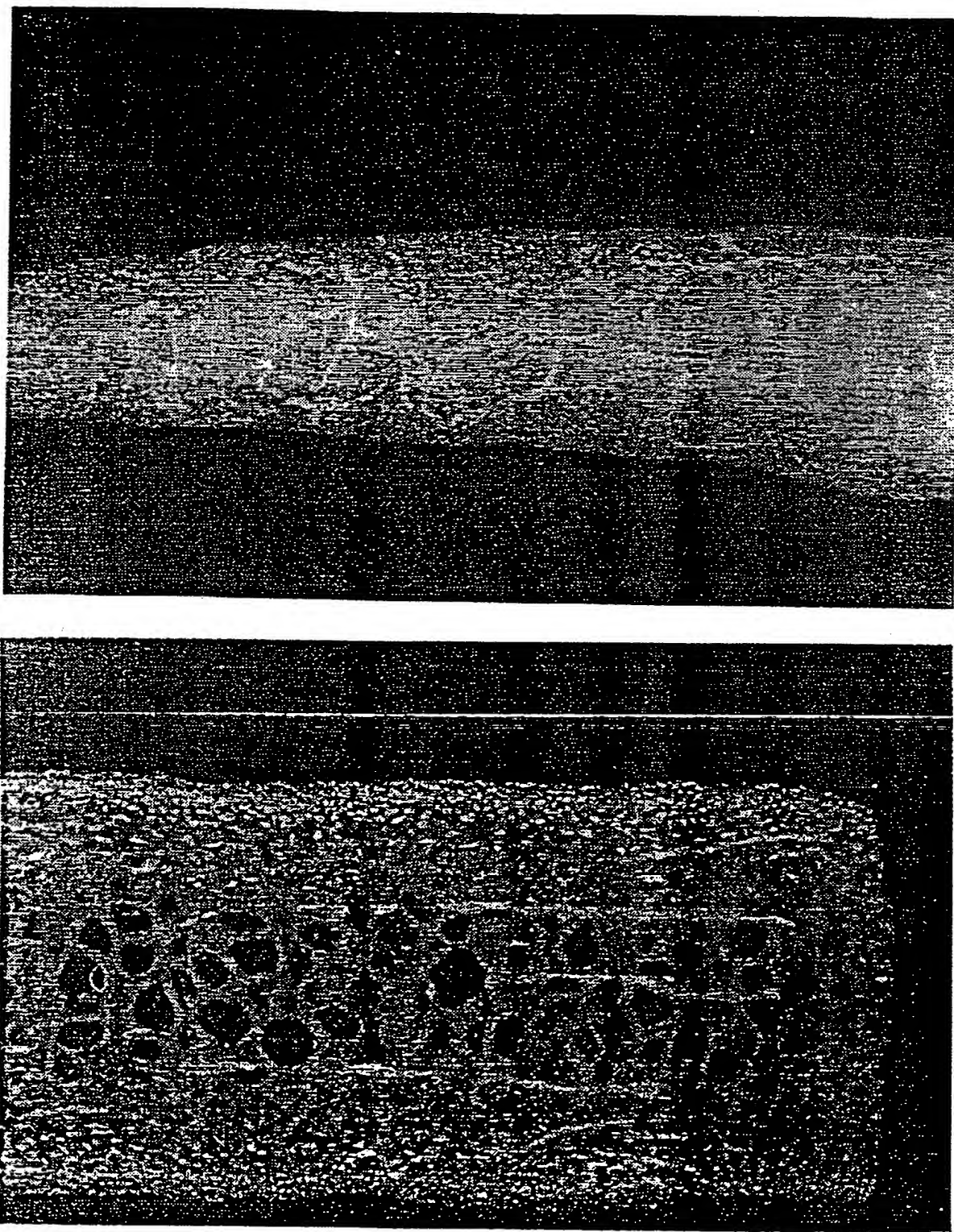


Fig.1. Calcofluor White stainings revealing fungi in upper part and lower part of *Gracilaria lemaneiformis*. (108x and 294x).

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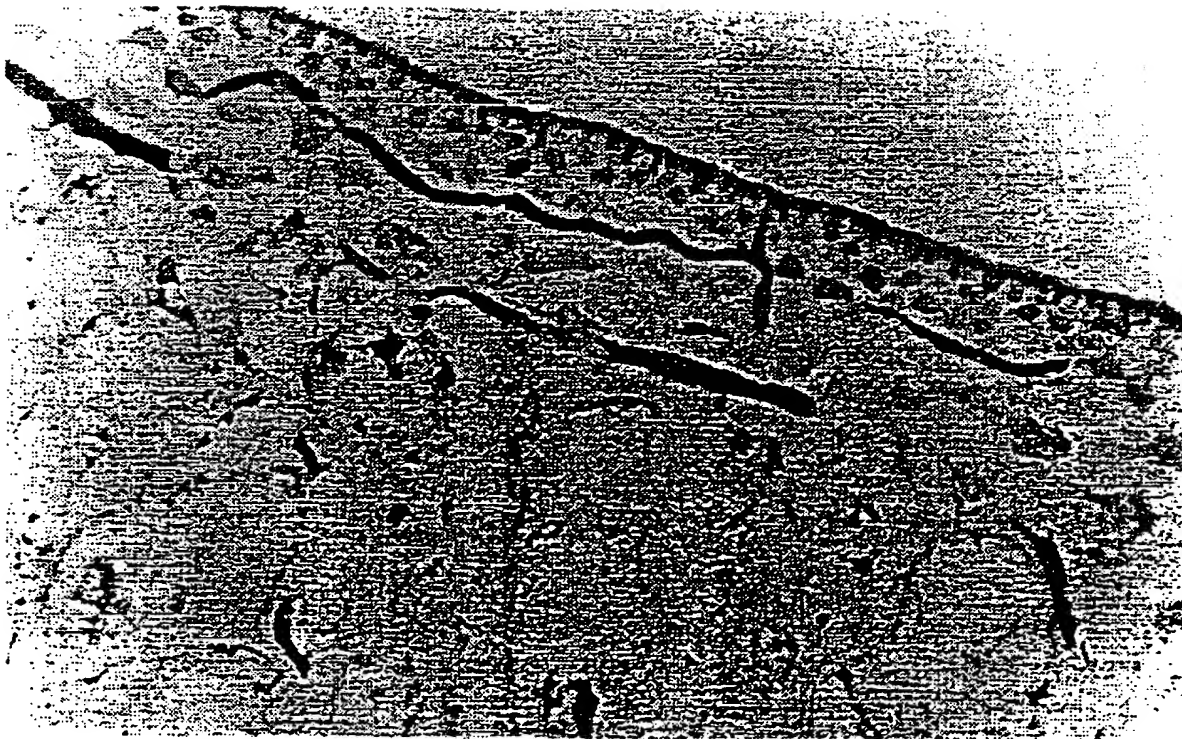


Fig.2. PAS / Anilinblue Black staining of *Gracilaria lemaneiformis* with fungi. The fungi have a significant higher content of carbohydrates.

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Fig. 3. The micrograph shows longitudinal and grazing sections of two thin-walled fungal hypha (f) growing between thick walls (w) of algal cells. Note thylacoid membranes in the algal chloroplast (arrows).

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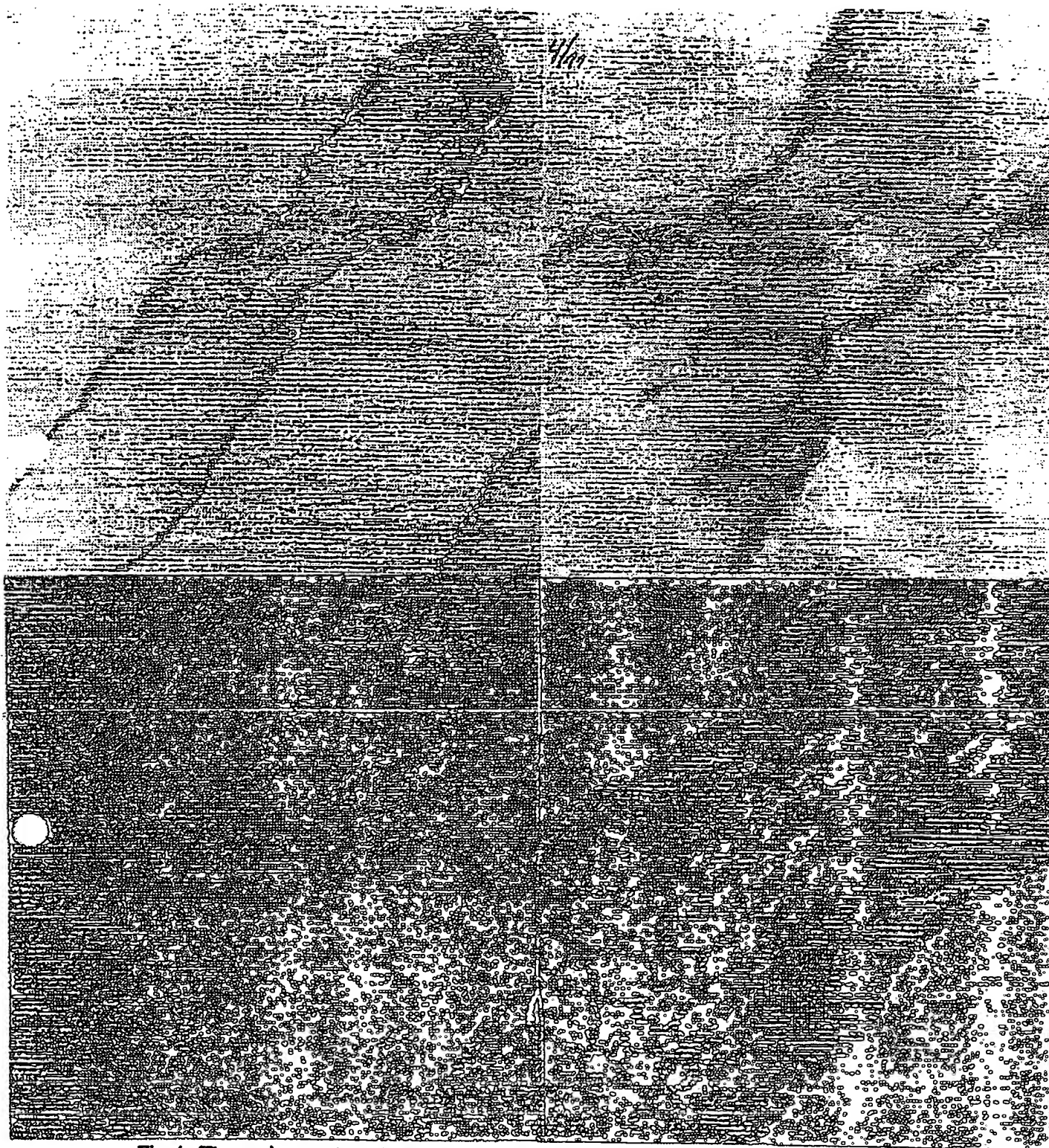


Fig.4. The antisense detections with clone 2 probe (upper row) are restricted to the fungi illustrated by the Calcofluor White staining of the succeeding section (lower row). (46x and 108x).

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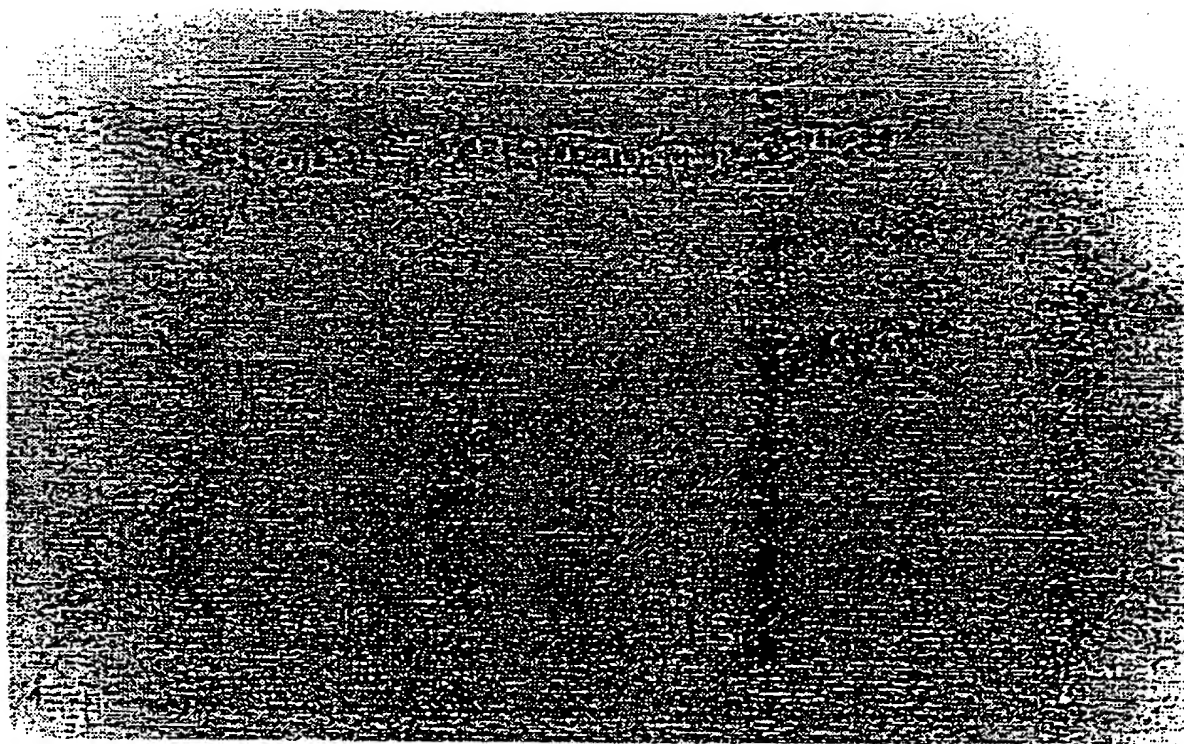
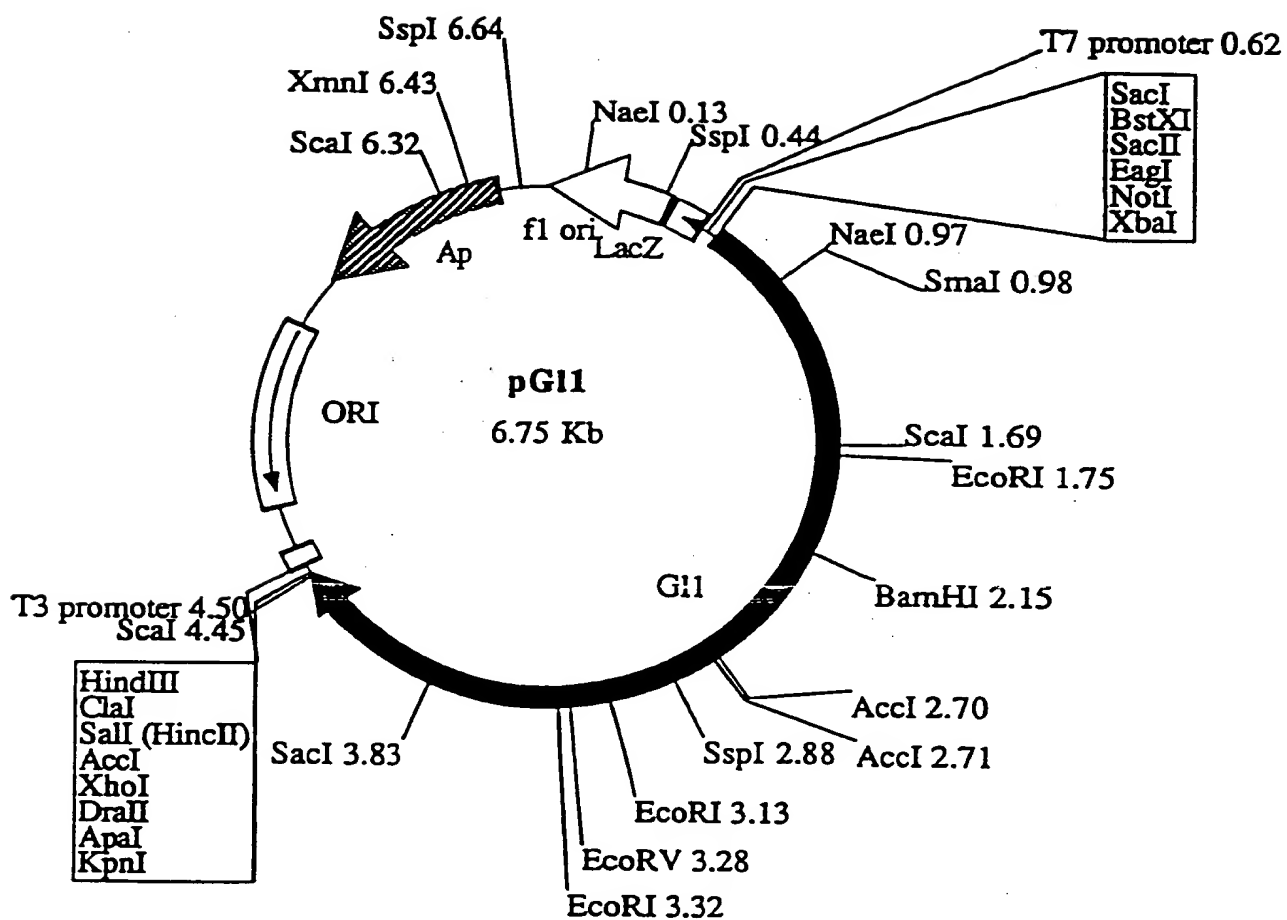


Fig.5. Intense antisense detections with clone 2 probe are found over the fungi in *Gracilaria lemaneiformis* (294x).

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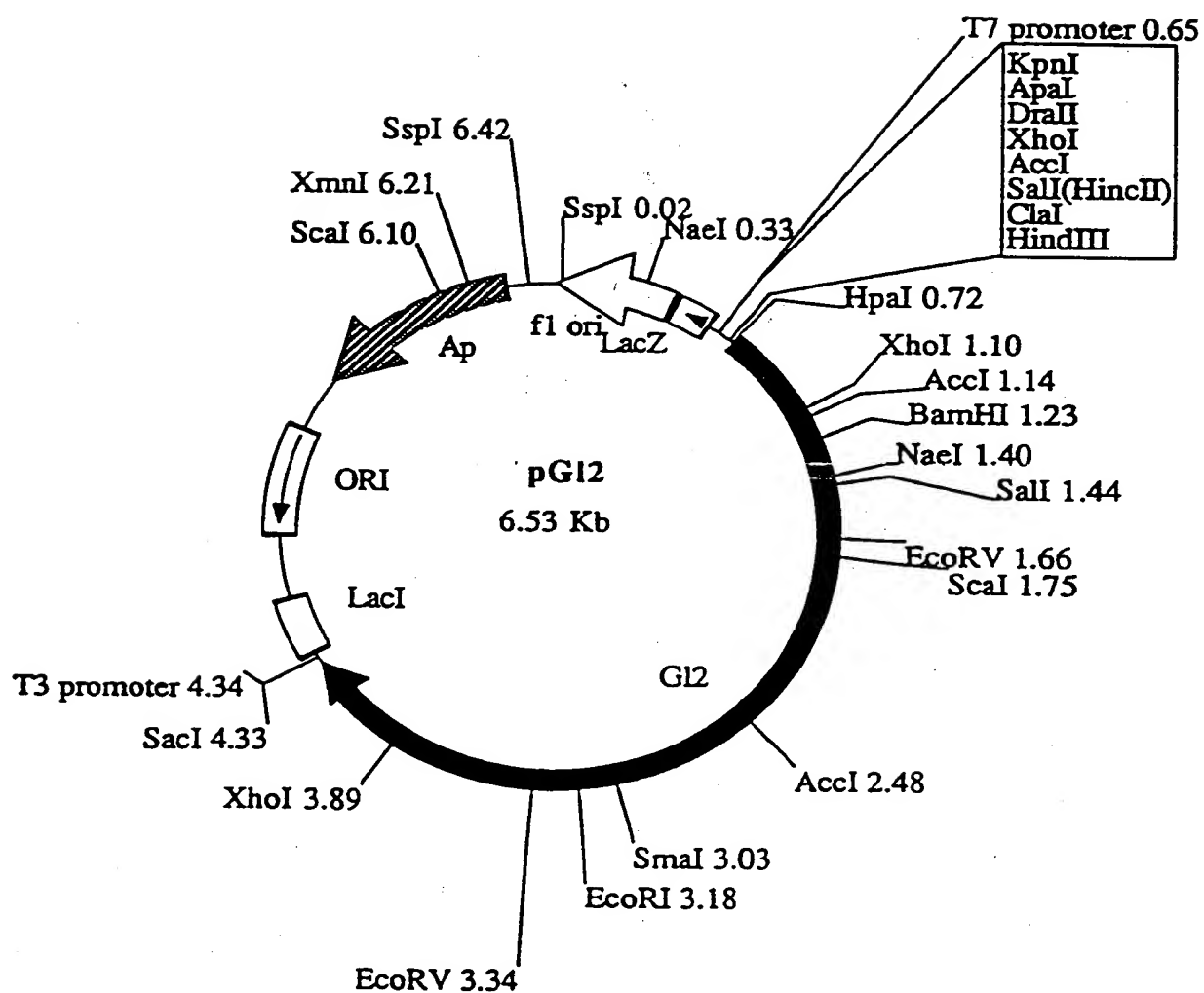
Fig. 6.



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Fig. 7.



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FIGURE 8

MFSTLAFVAP SALGASTFVG AEVRSNVRIH SAFFAVHTAT RKTNRNLNVSM TALSDKQTAT
AGSTDNPDGI DYKTYDYVGV WGFSPLSNTN WFAAGSSTPG GITDWTATMN VNFDRIDNPS
ITVQHPVQVQ VTSYNNNSYR VRFNPDGPIR DVTRGPILKQ QLDWIRTQEL SEGCDPGMTF
TSEGFLTFET KDLSVIIYGN FKTRVTRKSD GKVIMENDEV GTASSGNKCR GLMFVDRLYG
NAIASVNKNF RNDAVKOEGF YGAGEVNCKY QDTYILERTG IAMTNYNYN DN LNYNQWDLRP
PHHDGALNPD YYIPMYAAP WLIVNGCAGT SEQYSYGWFM DNVSQSYMNT GDTTWN SGQE
DLAYMGAQYG PFDQHFVYGA GGGMECVVTA FSLLQGKEFE NQVLNKR SVM PPKYVFGFFQ
GVFGTSSLLR AHMPAGENNI SVEEIVEGYQ NNNFPFEGLA VD VDMQDNLR VFTTKGEFWT
ANRVGTGGDP NNRSVFEWAH DKGLVCOTNI TCFLRNDNEG ODYEVNOTLR ERQLYTKNDS
LTGTDFGMDT DGPSDAYIGH LDYGGGVECD ALFPDWGRPD VAEWWGN NYK KLFSIGLDFV
WQDMTVPAMM PHKIGDDINV KPDGNWPNAD DPSNGQYNWK TYHPQVLVTD MRYENHGREP
MVTORNIHAY TLCESTRKEG IVENADTLTK FRRSYIISRG GYIGNQHFGG MWVGDNSTTS
NYIQMMIANN INMNMSCLPL VGSDIGGFTS YDNENQRTPC TGDLMVRYVO AGCLLPWFRN
HYDRWIESKD HGKDYQELYM YPNEMDTLRK FVEFRYRWOE VLYTAMYONA AFGKPIIKAA
SMYNND SNVR RAONDHFLLG GHDGYRILCA PVVWENSTER ELYLPVLTOW YKFGPDFDTK
PLEGAMNGGD RIYNYPVPQS ESPIFVREGA ILPTRYTLNG ENKSLNTYTD EDPLVFEVFP
LGNNRADGMC YLDDGGVTTN AEDNGKFSVV KVAAEODGGT ETITFTND CY EYVFGGPFYV
RVRGAQSPSN IHVSSGAGSQ DMKVSSATSR AALFNDGENG DFWVDOETDS LWLKLPNVVL
PDAVITIT

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GL1	-	MFSTLAFVAPSALGASTFVGAEV-RSNVRIHSAFPAVHTATRKTNRLNVS	-49
GL2	-	MYPTLTFVAPSALGARTFTCVGIFRSHILIHVVPAVRLAVRKS NRLNVS	-50
GL1	-	MTALSDKQTATAGSTDNDPDGIDYKTYDYGVWGFSPLSNTNWFAAGSSTP	-99
GL2	-	MSALFDKPTAVTGGKDNPDNINYYTTYDYVPVWRFDPLSNTNWFAAGSSTP	-100
GL1	-	GGITDWTATMNVNFDRIIDNPSITVQHPVQVQVTSYNNNSYRVRFNPDGPI	-149
GL2	-	GDIDDWTATMNVNFDRIIDNPSFTLEKPVQVQVTSYKNNCFRVRFNPDGPI	-150
GL1	-	RDVTRGPILKQQLDWIRTQELSEGCDPGMTFTSEGFLT FETKDL SVI IYG	-199
GL2	-	RDVDRGPILQQLNWIRKQE QSKGFDPKMGFTKEGFLKFETKDLNVI IYG	-200
GL1	-	NFKTRVTRKSDGKVI MENDEVGTASSGNKCRGLMFVDRLYGNAIASVNKN	-249
GL2	-	NFKTRVTRKRDGKGIMENNEVPAGSLGNKCRGLMFVDRLYGTAIASVNEN	-250
GL1	-	FRNDAVKQEGFYGAGEVNCKYQDT-----YILERTGIAMTNYNYDNLNY	-293
GL2	-	YRNDPDRKEGFYGAGEVNCEFDWSEQNRNKYILERTGIAMTNYNYDNYNY	-300
GL1	-	NQWDLRPPHHDGALNPDYIIPMYAAPWLIVNGCAGTS-EQYSYGWFMDN	-342
GL2	-	NQSDLIAP--GYSPDPNFYIIPMYFAAPWVVVKGCSGNSDEQYSYGWFMDN	-348
GL1	-	VSQSYMNTGDTTWN SGQEDLAYMGAQYGPFDQHFVYGAGGGMECVVTAFS	-392
GL2	-	VSQTYMNTGGT SWNCGEENLAYMGAQC GPFDQHFVYGDGDGLE DVVQAFS	-398
GL1	-	LLQKKEFENQVLNKR SVMPPKYVFGFFQGVFGTSSLLRAHMPAGENNISV	-442
GL2	-	LLQKKEFENQVLNKR AVMPKYVFGYFQGVFGIASLLREQRPEGGNNISV	-448
GL1	-	EEIVEGYQNNNFPFEG LAVDVDMQDNL RVFTTKGEFWTANRVGTGGDPNN	-492
GL2	-	QEIVEGYQSNNFPLEGLAVD VDMQDNL RVFTTKIEFWTANKVGTGGDSNN	-498
GL1	-	RSVF EWAHDKGLVCQT NITCFLRNDNEGQDYEVNQTLRERQLYTKNDSL T	-542
GL2	-	KSVF EWAHDKGLVCQT NVTCFLRNDNGGADYEVNQTLREKGLYTKNDSL T	-548
GL1	-	GTD FGMTDDGPSDAY IGHLDYGGGVECDALFPDWGRPDVAEWGNNYKKL	-592
GL2	-	NTNF GTTNDGPSDAY IGHLDYGGGGNCDALFPDWGRPGVAEWGDNYSKL	-598
GL1	-	FSIGLDFVWQDMTPAMMPHKIGDDINVKPDGNWPNADDP SNGQYNWKTY	-642
GL2	-	FKIGLDFVWQDMTPAMMPHKVGDAVDTRSPYGWPNENDPSNGRYNWKSY	-648
GL1	-	HPQVLVTDMRYENHGREPMVTQRNIHAYTLCESTRKEGIVENADTLTKFR	-692
GL2	-	HPQVLVTDMRYENHGREPMFTQRNMHAYTLCESTRKEGIVANADTLTKFR	-698
GL1	-	RSYIISRGGYIGNQHFGGMWVGDNSTTSNYIQMMIANINNMNSCLPLVG	-742
GL2	-	RSYIISRGGYIGNQHFGGMWVGDNSSSQRYLQMMIANIVNMNSCLPLVG	-748
GL1	-	SDIGGFTSYDNENQRTPTCTGDLMVRYVQAGCLLPWFRNHYDRWIESKD HG	-792
GL2	-	SDIGGFTSYDG---RNVCPGDLMVRFVQAGCLLPWFRNHYGR LVEGKQEG	-795
GL1	-	KDYQELYMYPNEMDTLRKFVEFRYRWQEVLYTAMYQNAAFGKPIIKAASH	-842
GL2	-	KYYQELYMYKDEMATLRKFIEFRYRWQEVLYTAMYQNAAFGKPIIKAASH	-845

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GL1 - YNND5NVRRAQNDHFLGGHGHDGYRILCAPVVWENSTERELYLPVLTQWYK -892
      : : : : : : : : : : : : : : : : : : : : : : : : : : : : : :
GL2 - YDNDNRNVRGAQDDHFLGGHGHDGYRILCAPVVWENTTSRDLYLPVLTQWYK -895

GL1 - FGPDPDTKPLEGAMNGGDRIYNYPVPQSESPFVREGAILPTRYTLNGEN -942
      : : : : : : : : : : : : : : : : : : : : : : : : : : : : : :
GL2 - FGPDYDTKRLDSALDGGQMIKNYSVPQSDSPFVREGAILPTRYTLDGSN -945

GL1 - KSLNTYTDEDPLVFEVFPLGNRADGMCYLDDGGVTTNAEDNGKFSVVKV -992
      : : : : : : : : : : : : : : : : : : : : : : : : : : : : : :
GL2 - KSMNTYTDKDPVFEVFPLGNRADGMCYLDDGGITDAEDHGKFSVINV -995

GL1 - AAEQDGGTETITFTNDCEYEVFGGPFYVRVRGAQSPSNIHVSSGAGSQDM -1042
      : : : : : : : : : : : : : : : : : : : : : : : : : : : : : :
GL2 - EALRKGVTTTTIKFAYDTYQYVFDGPFYVRIRNLTTASKINVSSGAGEEDM -1045

GL1 - KVSSATSRAALFNDGENGDFWVDQETDSLWLKLPNVVLPDAVITIT -1088
      : : : : : : : : : : : : : : : : : : : : : : : : : : : : : :
GL2 - TPTSANSRAALFSDGGVGEYWADNDTSSLWMKLPNLVLQDAVITIT -1091

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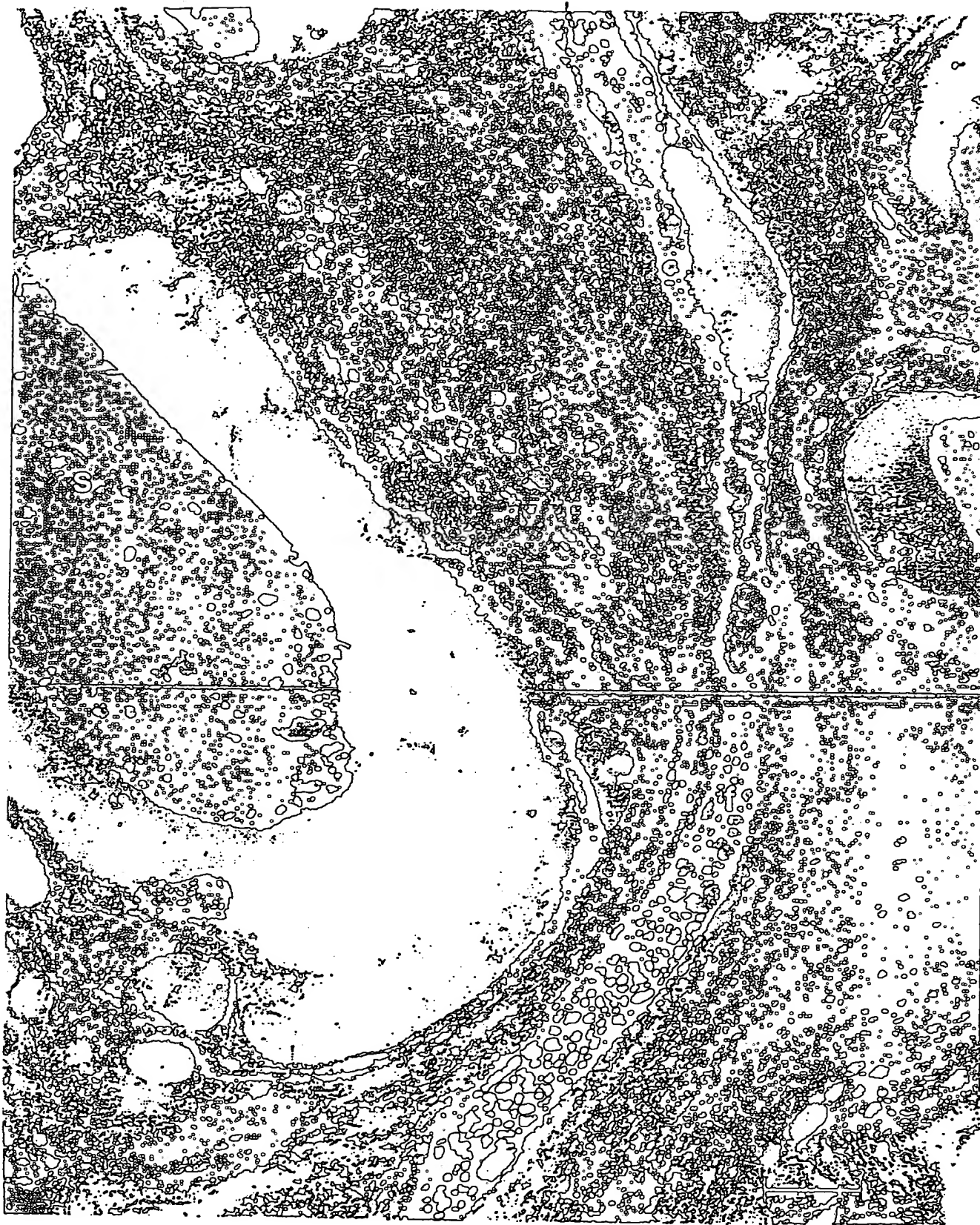


Figure 10. Microphotograph of a fungal hypha (f) growing between algal cell walls (w). Note grains of floridean starch (s) and thylakoids (arrows) in the algal cell. Bar = 2 μ m.

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INTERNATIONAL SEARCH REPORT

Internat'l Application No
PCT/EP 94/03399A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12N15/60 C12N9/88 C12N9/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 6 C12N C12P

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	FR,A,2 617 502 (SOCIÉTÉ NATIONALE ELF AQUITAINE) 6 January 1989 cited in the application see page 5, line 16 - line 33 see claims; examples ---	1-4
A	BIOCHIMICA ET BIOPHYSICA ACTA, vol.1156, no.3, March 1993 pages 313 - 320 S.YU ET AL 'Alpha-1,4-glucan lyase, a new class of starch / glycogen degrading enzyme. I. efficient purification and characterization from red seaweeds' cited in the application see page 314, left column, paragraph 3 - page 315, left column see page 315, right column, paragraph 3 --- -/--	1-5, 10, 11

☒ Further documents are listed in the continuation of box C.☒ Patent family members are listed in annex.

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Date of the actual completion of the international search

27 April 1995

Date of mailing of the international search report

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INTERNATIONAL SEARCH REPORT

Internat'l Application No

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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	PLANTA, vol.191, no.1, May 1993 pages 137 - 142 S. YU ET AL 'Alpha-1,4-glucan lyase, a new class of starch / glycogen-degrading enzyme. II. Subcellular localization and partial amino-acid sequence' see the whole document -----	1-5,10
P,X P,A	WO,A,94 09122 (ALGATECH AB) 28 April 1994 see page 6, line 5 - line 8 see page 11, line 24 - page 16, line 10 see claims -----	1-5,10 1

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No

PCT/EP 94/03399

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
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